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Subdivision M: Biorational Pesticides

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PESTICIDE ASSESSMENT GUIDELINES

Subdivision M

Biorational Pesticides

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As a guideline for the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), Subdivision M provides guidance on developing data on biochemical and microbial pest control agents to determine their fate in the environment and to evaluate potential adverse effects on humans and other nontarget organisms. These guidelines include further guidance on when data are required by 40 CFR Part 158, and contain the standards for conducting acceptable tests, guidance on evaluation and reporting of data, and examples of acceptable protocols. Subdivision M constitutes one volume of a twelve-part NTIS Federal Insecticide, Fungicide and Rodenticide Act guideline series.

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Foreword

Subdivision M describes protocols which may be used to perform testing on biochemical and microbial pest control agents to support their registration as pesticides under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). Protocols are provided for determining the chemical fate of these pesticides in the environment and for evaluating their potential adverse effects on humans and other nontarget organisms. Subdivision M is a nonregulatory companion to 40 CFR Part 158, Data Requirements for Registration. It has been the subject of comment at a series of public meetings, the last of which occurred in July 1982. Data requirements established by 40 CFR Part 158 are discussed in Subdivision M so that it can be read as a complete package and so that the testing procedures for biochemical and microbial pesticides can be explained in their proper context.

SUBDIVISION M: GUIDELINES FOR TESTING BIORATIONAL PESTICIDES

Table of Contents

DISCUSSION

Page

I. Scope of Subdivision M 1
II. Background of Subdivision M 5
III. Organization and Philosophy of Subdivision M 6
IV. Product Analysis 9
V. Toxicology 10
VI. Residue Analysis 31
VII. Nontarget Organism Hazard 33
VIII. Environmental Fate and Expression 77
IX. Product Performance 85
X. Experimental Use Permits 86
XI. Promoting Biorational Pesticides By Modifying Label Claims 86

GUIDELINES

Series 150: Overview, Definitions, and General Provisions

150-1 Overview 89
150-2 Definitions 89
150-3 General provisions 93
150-4 Reporting of data 96

Series 151: Product Analysis Guidelines

151-1 General Information 100
151-2 Through 151-9 [Reserved]

Subseries: 151A: Product Analysis Guidelines for Biochemical Pest Control Agents

151-10 Product identity and disclosure of ingredients 100
151-11 Manufacturing process 102
151-12 Discussion on the formation of unintentional ingredients 102
151-13 Analysis of samples 103
151-14 [Reserved]
151-15 Certification of ingredient limits 103
151-16 Analytical methods for certified limits 103

Table of Contents
(Continued)

Page

151-17 Physical and chemical properties 104
151-18 Submittal of samples 104
151-19 [Reserved]

Subseries 151B: Product Analysis Guidelines

151-20 Product identity and disclosure of ingredients 106
151-21 Manufacturing process 108
151-22 Discussion on formation of unintentional ingredients 108
151-23 Analysis of samples 109
151-24 [Reserved]
151-25 Certification of ingredient limits 109
151-26 Physical and chemical properties 109
151-27 Submittal of samples 110

Series 152: Toxicology Guidelines

152-1 General information 112
152-2 Through 151-9 [Reserved]

Subseries 152A: Toxicology Guidelines

Group A-1: Tier I Testing

152-10 Acute oral toxicity study 117
152-11 Acute dermal toxicity study 118
152-12 Acute inhalation toxicity study 119
152-13 Primary eye irritation study 120
152-14 Primary dermal irritation study 121
152-15 Hypersensitivity study 122
152-16 Hypersensitivity incidents 122
152-17 Studies to detect genotoxicity 123
152-18 Cellular immune response studies 125

Group A-2: Tier II Testing

152-19 Mammalian mutagenicity tests 129
152-20 Subchronic oral dosing studies 133
152-21 Subchronic dermal toxicity study 134
152-22 Subchronic inhalation toxicity study 135

Table of Contents
(Continued)

Page

152-23 Teratogenicity studies 136
152-24 Cellular immune response studies 137
152-25 [Reserved]

Group A-3: Tier III Testing

152-26 Chronic exposure study 139
152-27 Through 152-28 [Reserved]
152-29 Oncogenicity studies 140

Series 152B: Toxicology Guidelines for Microbial Pest Control Agents

Group B-1: Tier I Testing

152-30 Acute oral toxicity/infectivity study with microbial pest control 142
152-31 Acute dermal toxicity/infectivity study with microbial pest control agents 145
152-32 Acute inhalation toxicity/infectivity study with microbial pest control agents 149
152-33 Intravenous, intracerebral, and intraperitoneal toxicity/infectivity studies with microbial pest control agents 156
152-34 Primary dermal irritation study with microbial pest control agents 160
152-35 Primary eye irritation study with microbial pest control agents
152-36 Hypersensitivity study with microbial pest control agents 166
152-37 Hypersensitivity incidents with microbial pest control agents 167
152-38 Effects of microbial pest control agents on the cellular immune response system 168
152-39 Tissue culture tests with viral agents 170

Group B-2: Tier II Testing

152-40 Acute oral toxicity/infectivity studies with viral or protozoan agents 174

Table of Contents
(Continued)

Page

152-41 Acute inhalation toxicity/infectivity study with viral or protozoan agents 177
152-42 Subchronic oral dosing study with protozoa 180
152-43 Acute intraperitoneal or intracerebral toxicity/infectivity tests with bacteria, fungi, and protozoa 181
152-44 Primary dermal irritation study with microbial agents 182
152-45 Primary eye irritation study with microbial agents 183
152-46 Effects of microbial pest control agents on the cellular immune response system 183
152-47 Teratogenicity tests with viral agents 186
152-48 Bacterial and fungal virulence enhancement 186
152-49 Mammalian mutagenicity testing with microbial pest control agents 189

Group B-3: Tier III Testing

152-50 Chronic feeding study 190
152-51 Oncogenicity studies 190
152-52 Mutagenicity testing 191
152-53 Teratogenicity studies 192

Series 153: Residue Analysis Guidelines

153-1 Overview 192
153-2 [Reserved]
153-3 Residue data requirements for biochemical pest control agents 193
153-4 Residue data requirements for microbial pest control agents 194

Series 154: Nontarget Organism Hazard Guidelines

154-1 General Information 196
154-2 Through 154-5 [Reserved]

Subseries 154A: Nontarget Organism Hazard Guidelines for Biochemical Agents

Group A-1: Tier I Testing

154-6 Avian single dose oral toxicity test 210

Table of Contents
(Continued)

		Page
154-7	Avian dietary toxicity test	212
154-8	Freshwater fish acute bioassay	213
154-9	Freshwater aquatic invertebrate acute bioassay	215
154-10	Plant studies	217
154-11	Nontarget insect testing	219
Group A-2:	Tier III Testing	
154-12	Terrestrial wildlife testing	220
154-13	Aquatic animal testing	221
154-14	Plant studies	221
154-15	Nontarget insect testing	222
Subseries 154B:	Nontarget Organism Hazard Guidelines for Microbial Agents	
Group B-1:	Tier I Testing	
154-16	Avian single-dose oral toxicity and pathogenicity test	223
154-17	Avian injection pathogenicity test	226
154-18	Wild mammal toxicity and pathogenicity testing	229
154-19	Freshwater fish toxicity and pathogenicity testing	232
154-20	Freshwater aquatic invertebrate toxicity and pathogenicity testing	238
154-21	Estaurine and marine animal toxicity and pathogenicity tests	244
154-22	Plant studies	251
154-23	Nontarget insect testing for toxicity/pathogenicity to insect predators and parasites	252
154-24	Honey bee toxicity/pathogenicity tests	254
Group B-2:	Tier III Testing	
154-25	Terrestrial wildlife and aquatic organism toxicity testing	255
154-26	Long-term avian pathogenicity and reproduction test	256

Table of Contents
(Continued)

		Page
154-27	Definitive aquatic animal pathogenicity tests	260
154-28	Fish embryo-larvae studies and life cycle studies of fish and aquatic invertebrates	261
154-29	Aquatic ecosystem-pathogenicity tests	264
154-30	Special aquatic tests-tissue culture, microorganisms/stress interaction tests [Reserved]	
154-31	Plant studies	266
154-32	[Reserved]	

Group B-3: Tier IV Testing

154-33	Simulated and actual field testing for mammals and birds	267
154-34	Simulated or actual field testing for aquatic organisms	269
154-35	Simulated or actual field testing for insect predators and parasites [Reserved]	
154-36	Simulated or actual field testing for insect pollinators [Reserved]	

Series 155: Environmental Fate and Expression Guidelines

Subseries 155A: Tier II Environmental Fate Guidelines for Biochemical Pest Control Agents

155-1	General Information	272
155-2	And 155-3 [Reserved]	
155-4	Volatility	276
155-5	Dispenser - water leaching	277
155-6	Absorption - desorption	279
155-7	Octanol/water partition coefficient	279
155-8	Ultraviolet - visible absorption	280
155-9	Hydrolysis	281
155-10	Aerobic soil metabolism	281
155-11	Aerobic aquatic metabolism	282
155-12	Soil photolysis	283
155-13	Aquatic photolysis	284
155-14	[Reserved]	

Table of Contents
(Continued)

Page

Subseries 155B: Tier II Environmental Expression Guidelines for Microbial Agents

155-15 General information 285
155-16 General test standards 288
155-17 Reporting and evaluation of data 289
155-18 Tests to determine expression in a terrestrial environment 290
155-19 Tests to determine expression in a freshwater environment 294
155-20 Tests to determine expression in a marine or estaurine environment 297
155-21 Through 155-23 [Reserved]

Group 156: Product Performance Guidelines

156-1 General Provisions 299
156-23 Specific Provisions 300

Series 157: Experimental Use Permit Guidelines

157-1 Scope and intent 301
157-2 [Reserved]
157-3 General provisions 301
157-4 Specific provisions 301

Series 158: Label Development

158-1 Product label guidelines 304

I. SCOPE OF SUBDIVISION M

A. Contents of Subdivision.

Subdivision M provides guidelines for testing and information on data submission concerning the following eight section series and topics:

151 Product analysis
152 Toxicology
153 Residue chemistry
154 Nontarget organism hazards
155 Environmental fate and expression
156 Product performance
157 Experimental use permit data
158 Label development

Proposed rule, 40 CFR Part 158, specified the kind of data and information that must be submitted to EPA to support the registration of each pesticide under the Federal Insecticide, Fungicide and Rodenticide Act. The Agency intends to promulgate Part 158 as a final rule during 1983. This subdivision provides detailed information relating to the data requirements listed in 40 CFR § 158.165 including the conditions under which each data requirement is applicable, the standards for acceptable testing, stated with as much specificity as the current scientific disciplines can provide, and the information that should be included in a test report.

1. Scope of biorational pesticides. Biorational pesticides are a distinct group, inherently different from conventional pesticides. Some of the characteristics that typically distinguish biorational from conventional pesticides are their unique non-toxic mode of action, low use volume, target species specificity, and natural occurrence. Based on these characteristics, the Agency expects that many biorational pesticides pose lower potential risks than conventional pesticides. Therefore, these pesticides are subject to a different set of data requirements, as specified in §158.33. Biorationals are comprised of two major categories of pesticides: the biochemical pest control agents (e.g., pheromones, hormones, natural insect and plant growth regulators and enzymes) and the microbial pest control agents (e.g., microorganisms). The relationships between conventional pesticides, biological control agents, and biorational pesticides are illustrated in Figure 1. Pesticides to be included in these categories are determined as follows:

(2) The chemical must be naturally occurring, or if the chemical is synthesized by man, then it must be structurally identical to a naturally occurring chemical. For a synthetic chemical to be identical in chemical structure to a naturally occurring chemical,

the molecular structure(s) of the major component(s) of the synthetic chemical(s) must be the same as the molecular structures(s) of the naturally occurring analogue(s). Minor differences between the stereochemical isomer ratios (found in the naturally occurring compound compared to the synthetic compound) will normally not rule out a chemical being classified as a biorational unless an isomer is found to have significantly different toxicological properties than another isomer.

There are situations where a candidate chemical possesses many characteristics of a biorational pesticide, but does not technically meet the two criteria established for defining biochemical pest control agents. The Agency will evaluate chemical(s) that are substantially similar to biochemicals on a case-by-case basis to determine whether the chemical should be classified as a biorational or a conventional pesticide. For example, a case-by-case evaluation would be required if the exact molecular structure of the naturally occurring compound(s) is (are) unknown, or if the synthetic chemical is closely related to but not identical in structure to the naturally occurring compound, or if the mode of action is different in the target, compared to non-target organisms.

In these case-by-case situations, the criteria the Agency will use to determine whether the chemical(s) is (are) a biorational pesticide(s) include: 1) the chemical and toxicological significance of the differences in chemical structure, 2) the mode of action of the synthetic analogue in the target species as compared to the mode of action of the naturally occurring compound, and 3) differences in toxicity (as demonstrated in at least the Tier I screening tests for biorational pesticides, as specified in 40 CFR § 158.165) between the naturally occurring chemical and the synthetic analogue. If a synthetic analogue is found to demonstrate direct toxicity to any non-target organisms, based on Tier I testing, then the analogue may or may not be classified as a biorational pesticide.

In evaluating these case-by-case situations, the Agency may find it appropriate to classify a chemical as a biorational, yet still impose certain conventional pesticide data requirements for some disciplines (e.g., non-target organisms and environmental fate) and the biorational pesticide data requirements for the remaining disciplines.

Biochemical agents fall into four general biologically functional classes: semiochemicals, hormones, natural plant regulators, and enzymes. They are discussed below.

Semiochemicals. Chemicals emitted by plants or animals that modify the behavior of receptor organisms of like or different kinds are termed semiochemicals. They include pheromones, allomones, and kairomones. Pheromones are substances emitted by members of one species that modify the behavior of others within the same species. Allomones are chemicals emitted by one species which modify the behavior of a different species, to the benefit of the emitting species. Kairomones are chemicals emitted by one species which modify the behavior of a different species to the benefit of the receptor species.

Hormones. Hormones are biochemical agents are are synthesized in one part of an organism and translocated to another where they have controlling, behavioral, or regulating effect.

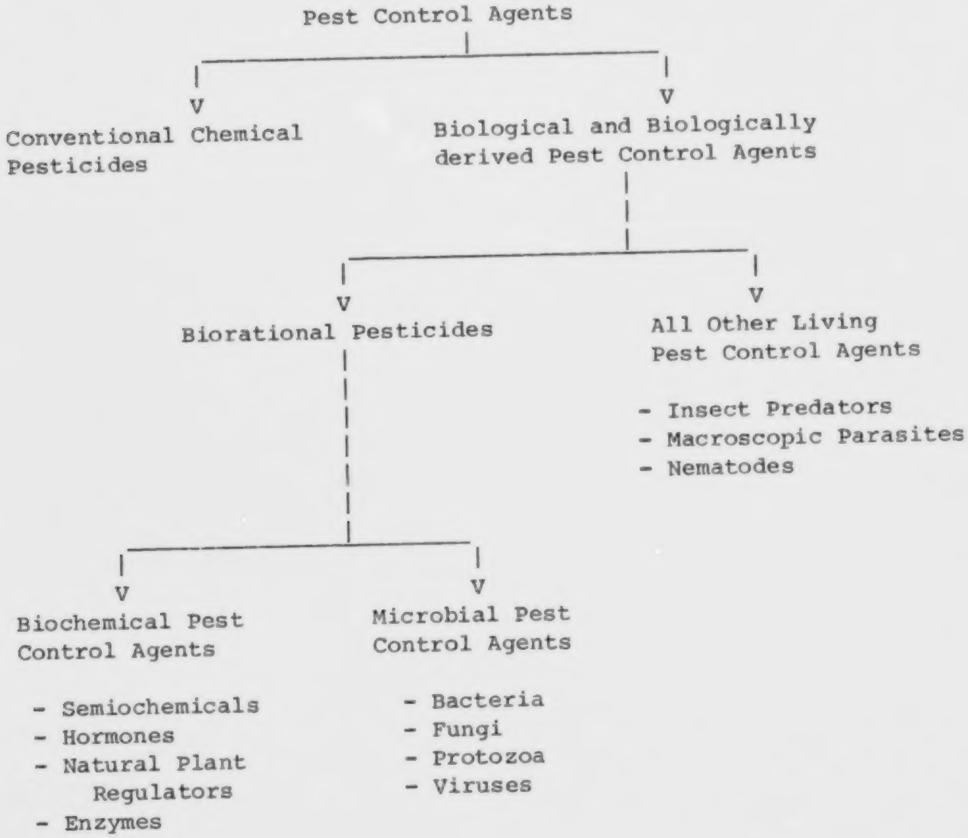
Natural plant regulators. Natural plant regulators are chemicals produced by plants that have toxic, inhibitory, stimulatory, or other modifying effects on the same or other species of plants. Some of these are termed "plant hormones" or "phytohormones."

Enzymes. For the purposes of this subdivision, enzymes are protein molecules that are the instruments for expression of gene action and that catalyze chemical reactions.

(b) Microbial pest control agents. The biorational pesticides referred to as microbial pest control agents include (but are not limited to) bacteria, fungi, viruses, and protozoans. The guidelines apply to all microbial pest control agents used as pesticides, including not only those that are naturally occurring, but also those that are strain-improved. Each variety or subspecies of a microbial pest control agent should be tested. Data necessary to support the registration of genetically-engineered microbial pest control agents will be determined by the Agency on a case-by-case basis except where specific requirements are specified in Part 158.

Pest control organisms such as insect predators, nematodes, and macroscopic parasites are not considered biorational pesticides, and are exempt from the requirements of FIFRA as authorized by sec. 25(b)(1) of FIFRA and specified in the Exemption from Regulation of Certain Biological Control Agents published in the Federal Register of June 2, 1982 (47 FR 23928).

Figure 1--RELATIONSHIPS BETWEEN CONVENTIONAL PESTICIDES, BIOLOGICAL CONTROL AGENTS AND BIORATIONAL PESTICIDES


Pest Control Agents
Conventional Chemical Pesticides
Biological and Biologically derived Pest Control Agents
Biorational Pesticides
All Other Living Pest Control Agents
- Insect Predators
- Macroscopic Parasites
- Nematodes
Biochemical Pest Control Agents
- Semiochemicals
- Hormones
- Natural Plant Regulators
- Enzymes
Microbial Pest Control Agents
- Bacteria
- Fungi
- Protozoa
- Viruses

II. BACKGROUND OF SUBDIVISION M

A. General.

EPA proposed the introduction, product chemistry, environmental chemistry, and fish and wildlife guidelines as Part 163 of Title 40 in the Code of Federal Regulations published in the Federal Register of July 10, 1978 (43 FR 29696). In the 1978 proposed guidelines, § 163.40-4 of the Introduction to the Guidelines (Subpart B) set forth EPA's policy for registering biological control agents as follows: "The data requirements for those living organisms or viruses which are pesticides will be determined on a case-by-case basis after consultation with the Agency."

EPA issued "Regulation of 'Biorational' Pesticides; Policy Statement and Notice of Available Background Document" published in the Federal Register of May 14, 1979 (44 FR 23994). In this statement of proposed policy, EPA defined biorational pesticides to include biological pest control agents and certain naturally occurring biochemicals which are inherently different in their mode of action from most organic and inorganic (i.e., conventional) pesticide compounds currently registered with EPA. The statement also presented EPA's intent to develop, in the next 24 months, guidelines setting forth the human health and environmental safety data requirements for the registration of biorational pesticides.

These guidelines provide information regarding the conduct of acceptable tests, guidance on evaluation and reporting of data, further guidance on when data are required, and examples of protocols. In addition, scientific publications are cited in the guidelines to provide useful information for designing test protocols.

B. Formulators' Exemption.

In the Preamble to the 1978 proposed Guidelines, EPA asked for public comment on the question of which data requirements should be extended to manufacturing-use products. After serious consideration of this issue, the Agency concluded that extending the data requirements to such pesticides is appropriate. The Agency was influenced by the views of commenters on this issue who generally favored a data submission requirement which makes the basic manufacturer of an active ingredient responsible for providing most of the data.

Therefore, a section of 40 CFR Part § 158, entitled "Formulators' Exemption" (§158.50) requires a registrant of a manufacturing-use product to submit (or cite) any data pertaining to the safety of an active ingredient in its product if the same data are required to support the registration of an end-use product that could legally be produced from the registant's manufacturing-use products. (An end-use product is a pesticide product bearing label directions for immediate end-use as a pesticide.) Section 158.50 also provides that such data must be submitted by an applicant for registration of the end-use product, except that the producer of the end-use product will generally not have to submit or cite data pertaining to registered products which the end-use producer purchases and uses to formulate the end-use product. This decision reflects the Agency's expectation that manufacturing-use product registrants will be the major source of registration data, and that end-use product formulators will, in most cases, need to supply much less data. This decision is consistent with the provisions of, and Congressional intent behind, sec. 3(c)(2)(D) of FIFRA, which provides that:

> No applicant for registration of a pesticide who proposes to purchase a registered pesticide from another producer in order to formulate such purchased pesticide into an end-use product shall be required to--
>
> (i) submit or cite data pertaining to the safety of such purchased product; or
>
> (ii) offer to pay reasonable compensation otherwise required by [§ 3(c)(1)(D)) of FIFRA] for use of any such data.

Implicit in sec. 3(c)(2)(D) is Congress' expectation that it would be the registrant of the manufacturing-use product who would provide significant amounts of data pertaining to the safety of its product. (See, e.g., Sen. Rep. No. 334, 95th Cong., 1st Sess., pp. 8-9.)

Moreover, if data requiarements were imposed solely on registrants of end-use products, sec. 3(c)(2)(D) might be read to prevent the Agency from obtaining data on the grounds that the data pertain to the safety of a purchased product.

III. ORGANIZATION AND PHILOSOPHY OF SUBDIVISION M

B. Approach to Testing.

The approach taken in developing these guidelines was significantly influenced by EPA's proposed policy (44 FR 23994) that states:

> In regulating biorational pesticides EPA will recognize that biorational pesticides are inherently different from conventional pesticides, and will take steps to substantiate by scientific data the expectation that many classes of biorational control agents pose lower potential risks than conventional pesticides. Although biorational pesticide registrants will not be relieved of the burden

of proof of their safety, the Agency will take into account the fundamentally different modes of action of biorationals and the consequent lower risks of adverse effects from their use.

The most important inherent differences between biorational pesticides and conventional pesticides are: target species specificity, generally nontoxic mode of action, and natural occurrence of the biorational agents. These factors have made the use of many biorationals practicable only under the direct supervision of a skilled entomologist, plant pathologist, weed scientist or integrated pest management (IPM) consultant. IPM techniques maximize usage of natural pest controls and cultural practices; therefore all introduced pest control materials (biorationals as well as conventional chemical pesticides) are used with discretion. These factors provide the basis for the Agency's expectation that many classes of biorational pest control agents pose a lower potential hazard than conventional pesticides and support the approach to testing discussed in the following paragraphs.

Three elements form the basis of EPA's approach and meet the intent of the above policy. They are: exposure criteria (for biochemicals), maximum hazard testing, and a tier testing scheme.

1. Exposure criteria. Certain factors often associated with biochemical pest control agents or their use, significantly limit the agent's potential for human and other nontarget organism exposure and, therefore, hazard. One or all of these factors provide a basis for criteria for reduced data requirements. These criteria are: low exposure pesticide formulation, low rate of application, nonaquatic use site, and high volatility. These criteria are described below.

(a) Low exposure pesticide formulation. Certain biochemicals are formulated in passive dispensers such as hollow fibers, tape, or fixed traps. The likelihood of oral or dermal human exposure and direct exposure of other nontarget organisms is low when pesticides are formulated in this manner.

(b) Low rate of application. Certain biochemical pesticides (e.g., semiochemicals) will be used in the field at very low rates. Low use rates can be tentatively described as 0.7 ounces (20 grams) or less active ingredient per acre per application, with rates of 1 to 5 grams per acre more common. Such low rates of application result in equally low and possibly nonhazardous levels of exposure to humans and other nontarget organisms. These figures are based on label information of currently registered pheromones and on the labels of those still under experimental use permits.

(c) Non-aquatic use site. Biochemical pest control agents applied on land pose less of a risk to nontarget aquatic species than those applied directly to water. Therefore, biochemical agents applied directly to land are more likely to qualify for reduced testing for nontarget organism hazard than those applied directly to water.

(d) High volatility. High volatility is a physical characteristic of some biochemicals that for terrestrial use sites would almost preclude the potential for aquatic exposure and would reduce the likelihood for residues on food or feed crops and residues on terrestrial animal food, e.g., vegetation, invertebrates.

2. Maximum hazard testing. The concept of maximum hazard testing is used in both the toxicology and nontarget organism sections. The Agency includes information in Tier I that reflect a maximum hazard approach to testing. The concept of maximum hazard testing is that the most challenging exposure in terms of the treatment dose or concentration, route of administration, and the age of test animals is used in the first tier of testing. Using this approach, the Agency believes that negative test results from testing under this approach would provide a high degree of confidence that no adverse effects would be likely to occur from the use of the biorational pest control agent.

3. Tier testing scheme. Four of the major section series, Residue Chemistry, Toxicology, Nontarget Organism Hazard, and Environmental Fate and Expression, use a tier testing scheme to ensure that only the minimum data necessary to make a scientifically sound regulatory decision are developed. This scheme eliminates the need for submittal of extensive data for those pesticides that are determined to be safe on the basis of Tier I data. The Agency believes many biorational pesticides will require only Tier I testing. The tier testing scheme is discussed in detail under paragraph III D of this Discussion.

B. Summary of Major Issues.

Major issues are identified and discussed in various sections throughout this Discussion. The following list itemizes each issue and indicates the location of each corresponding explanation in this Discussion.

- State of the art in safety testing protocols for microbial agents: V.B.1.; VII.B-1.2(f); VII.B-2.3(i); VII.B-3.3(b);

- Potential hazards unique to microbial pest control agents: V.B.4(a)&(b); VII.B-1.2(e); VI.B-3.3(d)-(f);

- Residue analysis procedures and tolerance setting for microbial agents: VI.B.;
- Unresolved questions pertaining to Tier I test protocol for nontarget organisms: VII.A-2.3(a)&(b); VII.B-1.2(a)-(d); VII.B-2.3(b)-(h); VII.B-3.3(a);
- Use of in vitro testing for nontarget organism hazard assessment: VII.B-2.3(a); VII.B-3.3(c);
- Promoting biorational pesticides by modifying label claim requirements: XI.

IV. PRODUCT ANALYSIS

A. Biochemical Pest Control Agents.

The product analysis data for biochemical pest control agents (§§ 151-10 through -18 of this subdivision) closely parallel those for conventional chemical pesticides as specified in Subdivision D (§§ 163.60-1 through 64-1.) Both Subdivisions D and M of these guidelines solicit detailed information on the procedures by which the active ingredient is produced, and the techniques used to ensure a uniform or standardized product. Refer to §§ 151-11(a)(ii), (iii), (iv) and 151-12. If the standardization techniques include methods of bioassay, then these methods should be described. The Agency is particularly interested in the ingredients which may be toxic or sensitizing to humans or other nontarget species.

B. Microbial Pest Control Agents.

The product analysis data for microbial pest control agents under §§ 151-20 through -26 of this subdivision, to some extent, parallel those for conventional chemical pesticides as specified in Subdivision D. However, due to the unique nature, composition, and mode of action of the microbial agents, there are some important differences. For example, protozoa, bacteria, fungi, and viruses should be identified to the extent possible by taxonomic position, serotype, composition, and strain, or by any other appropriate specific means. This information would take the place of chemical name and structural formula information for conventional pesticides. As a result, the guidelines in §§ 151-20 through -26 generally reference the corresponding section in Subdivision D and indicate those portions of Subdivision D which do not apply.

In addition, the Agency must be reasonably assured that the methods used and the data submitted are capable of demonstrating that the biorational pesticide used in the field is the same as that which was tested for safety.

V. TOXICOLOGY

Biorational pesticides affect pest populations by controlling physiological processes, by altering behavior, by competing for space and nutrients, by parasitizing and lysing the pest, or by replicating in an infective process to cause disease so that the pest is destroyed. Compared to most conventional pesticides, they act in very small amounts when used in the field. However, in contrast to the usual concentrations of these agents found in the environment, some of these agents will be distributed or strategically placed in the environment in relatively high amounts for short periods of time. The testing for registration of the product and the kinds of data developed must be sufficient to allow scientific experts to assess the potential hazards associated with the use of biorational pesticides.

The Agency was greatly assisted in developing the guidelines for human hazard evaluation testing for biorational pesticides by work performed by the American Institute of Biological Sciences (AIBS) under Grant No. R806461. The report from this grant (Human Hazard Evaluation Testing for Biorational Pesticides) served as the basic document for this toxicology section.

The major concerns of the Agency regarding biorational pesticides with respect to toxicology are:

- (1) "infectivity" - the potential for the microorganism to survive and replicate in a human host. Related concerns include persistence, invasiveness, colonization, and other host-parasite interactions.

- (2) "virulence-toxicity" - the potential for direct injury at the cellular, tissue, or organ level. Included are the long term effects associated with oncogenicity, carcinogenicity, and teratogenicity.

- (3) "hypersensitivity" - an immune response leading to an abnormal sensitivity. Serious reactions include allergies and anaphylaxis.

These concerns must be addressed in terms of the potential impact of these agents on the population as a whole, not only with respect to the normal individual but also with respect to persons with altered defenses who might encounter these agents, and who represent a subpopulation at higher risk. At present, the viruses are of particular concern because they generally exhibit a greater incidence of genetic change than other living forms. More intensive testing related to this characteristic, therefore, is indicated for the viruses.

The test batteries should provide negative data or data meeting specified limit criteria to assure the Agency that adverse effects would not result from the use of the agents. Because this conclusion must ordinarily be reached in order to register a biorational agent and because of the economics involved, the most desirable and practical approach to testing involves the tier concept. In general, testing beyond the first tier would be instituted when data demonstrating the potential for adverse effects are generated in the first Tier. It is recognized that for some biorational pesticides there are no well-recognized and standard test methods for assessing the toxicological hazards to mammals. Moreover, the testing method employed may vary among pesticide products simply because of the characteristics of the active agent. For example, it may be virtually impossible to conduct experiments exposing animals to aerosols of some fungal preparations because of the size of the fungal spore or mycelial element, the recovery rate for the test organism, the nature of the formulated product, or the concentration of the active material in the formulated product.

In the development of biorational pesticides, many problems related to hazard evaluation undoubtably will be encountered simply because the field is new. Much innovative research is required, and the standard methodology used with conventional pesticides is not easily adapted to some of the biorational agents. When problems arise, the registrant is urged to discuss the matter with the Agency so that alternative methods and protocols can be considered prior to the actual conduct of the tests.

Tables 3 and 4 under § 152-1 summarize the testing for biorational agents using the tier approach. Details are provided in §§ 152-1 through -53 of the guidelines.

A. Biochemical Pest Control Agents.

1. Introduction. Two basic assumptions served as the foundation upon which the biochemical testing regimen for evaluating human hazard potential was developed. First, an exemption from testing

based on extremely low exposure alone is not permitted by the Agency; second, standard chemical toxicity testing, as for the registration of conventional organic chemical pesticides, will generally apply to biochemicals considered for use as biorational pesticides.

2. Approach. The following general characteristics of biochemical pest control agents and their uses were considered in developing the safety evaluation testing protocols:

- (1) These compounds are generally not innately toxic, and their pesticidal action is not the result of target organism toxification.

- (2) In general, semiochemicals will be used in the field at very low use rates compared to conventional chemicals. Hormones or enzymes may be used at higher rates than semiochemicals, but use rates, in terms of active pesticide ingredient, will also generally be low. Therefore, human exposure to these products will be generally lower than human exposure to conventional pesticides.

- (3) In many instances (for semiochemicals especially), the site of physiological activity in the target pest will have no analogous site in non-target organisms or mammalian systems. Even in the case of enzymes, substrate sites may be species specific, and transport of enzyme in an active form to an intracellular or internal nontarget organ site following dermal, oral, or inhalation exposure is of low probability.

- (4) The biochemicals to be considered will be of known structure and, in most instances, information on the metabolic pathways for their synthesis and degradation will be available. Predictability of lack of formation of potentially toxic metabolites is highly probable: for example, degradation products of enzymes are generally without significant toxic potential. Although some peptides may be physiologically active, any toxicity associated with these breakdown products would be reflected in actual tests performed *in vivo*.

Pheromones isolated and purified from a single insect species may consist of a number of organic compounds, usually structurally related with similar properties. If the degradation products of the pheromone mixture have been chemically and physically characterized, and display little or no evident toxicity on the basis of available data, then it would not be necessary to conduct toxicity testing for each individual component of the naturally occurring mixture.

Conceivably, it may be desirable to combine several pheromone mixtures into a single product in order to broaden the target species, to enhance stability, or to provide other benefits to the user. In such instances, each pheromone complex should be toxicologically tested for possible adverse effects. In addition, the formulated product containing mixtures of complex pheromones may be tested.

As with all biorational pesticides, the concept of maximum hazard testing is used early in the tier testing regimen. The concept of maximum hazard testing is that the most challenging exposure in terms of route of exposure, species and age of test animal, dose administered, and similar factors, will be used in Tier I to identify any potentially hazardous agents. The Agency recognizes that the use of the maximum hazard testing approach will require flexibility in determining the appropriate and feasible dose(s) and route(s) of administration.

If the studies conducted in Tier I provide only negative data, no further testing is indicated. If, however, a potentially adverse effect is detected in maximum challenge experiments, the tests proceed through the tiers until the actual hazard of the agent can be evaluated and quantified.

In the first series of tests, data regarding acute toxicity, irritation, hypersensitivity, mutagenicity, and effects on cellular immune response are generated. Subsequent tests include subchronic and chronic exposures of animals, teratogenicity, oncogenicity and additional tests on mutagenicity and cellular immune response.

Appropriate controls must be tested to ensure that any toxicity or lethality is due to the presence of the active ingredient in the formulated product. Insofar as possible, the control for the test material should be identical to the formulated product except that it should be biologically inactive. Treatment by mild physical or chemical agents to inactivate the product or substitution of a similar but innocuous and inactive chemical for the active compound may provide a suitable control preparation for test.

3. Testing to address the major human safety concerns. The major concerns of the Agency for humans and domestic animals regarding the use of biochemicals as biorational pesticides are their acute toxicity, possible irritation or sensitization, and potential for mutagenic, teratogenic, or oncogenic activity, and effects on the cellular immune response system.

Acute toxicity determinations should be performed using the manufacturing use product, the technical grade of the active ingredient, and/or the formulated end-use product with test animals exposed by the oral, dermal, and inhalation routes. These acute toxicity tests, when supplemented with tests on irritancy, hypersensitivity, and cellular immune responses, should provide the necessary information to assess the toxicity hazard.

The potential for dermal and ocular irritancy, particularly of the formulated product, needs to be assessed in order to protect people handling the product. The following studies are presently considered sufficient to characterize this potential:

- (1) Primary skin irritation tests in laboratory animals by use of patch tests on intact and abraded skin, and, in certain instances, tests of irritation of the eye; and

- (2) Observations in exposed people during laboratory testing, pilot production, handling, transport, and field trials of the product.

A biochemical pest control agent, especially a protein or hapten, may induce allergic responses. In order to develop adequate label precautions for handling the material, the registration applicant should investigate the biochemical's allergenicity potential. The studies include sensitivity testing in experimental animals, and the species selected should be known to give some prediction of the potential of the product or its ingredients to cause immune disorders. In addition, the registrant should report and submit any observations of such effects as skin sensitivity, respiratory distress, or allergic symptoms in people handling the material during its development or production.

Many well-established _in vitro_ immunological techniques are available that can provide information as to whether a substance affects the immune response. Such _in vitro_ tests often correlate well with _in vivo_ results. Positive correlations between _in vitro_ and _in vivo_ results have been observed with a wide variety of antimetabolites (i.e., protein and nucleic acid-inhibiting substances), x-irradiation, and corticosteroids. For example, almost all agents which suppress the antibody-forming ability of lymphoid cells _in vitro_ have similiar effects _in vivo_.

The Agency is providing a tier system to ascertain the potential immunological hazard of the biochemical agents for humans. This testing system measures the effect of the agent on immunocompetence with both _in vivo_ and _in vitro_ tests including measurements of blood cell count, leukocyte responses (T and B cell numbers), functional activity of blood leukocytes, macrophage number and function, serum protein levels, antibodyforming activity, and cellular immune responses.

Information generated by the immune response tests will also be of value as a screening procedure for carcinogenicity, since many immunomodulating agents are also carcinogens. The testing scheme described above reveals not only carcinogens which affect lymphoid cells _per se_, but also agents which have other carcinogenic activity.

4. Evaluating potential for oncogenic and mutagenic effects. These guidelines provide a comprehensive testing scheme to assess the potential oncogenic and mutagenic effects of each biochemical pest control agent. This scheme consists of three parts:

- (1) Microbial assays in Tier I and short-term mammalian assays in Tier II;
- (2) Cellular immune response studies in Tiers I and II; and
- (3) An oncogenicity study in Tier III.

All three parts of the testing scheme could apply, depending on the biochemical agent, its use pattern, expected human exposure, and results of certain other studies.

The approach recommended by a panel of American Institute of Biological Sciences (AIBS) scientists would be to require only a microbial bioassay (Ames test) and cellular immune response studies in Tier I, to require the oncogenicity testing in Tier II, and to require a chronic (oral, dermal, or inhalation) study in Tier III.

The approach proposed in the September 29, 1980 draft of these guidelines, and subsequently endorsed by the FIFRA Scientific Advisory Panel (SAP) in October, 1980 was to require:

- (1) A battery of five mutagenicity studies in Tier I consisting of:
  - (a) A bacterial assay for reverse gene mutation (Ames assay);
  - (b) A mammalian cell point mutation assay _in vitro_;
  - (c) A prophage induction assay in lysogenic bacteria;
  - (d) A mammalian _in vivo_ cytogenetic assay, and;
  - (e) Either a DNA damage/repair assay, or an assay for mitotic recombination.
- (2) Cellular immune response studies in Tiers I and II; and
- (3) Oncogenicity testing at Tier II or III.

After careful consideration of the SAP's comments and the written and oral comments from industry, the Agency decided to include a somewhat modified approach to mutagenicity testing as described above. This modified approach is consistent with the SAP's comments and also responds to industry's request to reduce the economic impact of the mutagenicity testing requirements.

The cellular immune response studies will serve as screens that identify potential carcinogenic agents, as well as provide information on effects of the immune response system. The second set of cellular immune response studies would be located in Tier II.

Testing for oncogenicity in mammalian species would apply only if the following conditions are met:

- (1) If the biochemical agent produced, in Tier II subchronic studies under §§ 152-19 through -21, a morphological effect, e.g., hyperplasia or metaplasia, in any organ that potentially could lead to neoplastic change; or
- (2) If adverse effects suggesting oncogenic potential are observed in the Tier II cellular immune response studies or short term mammalian mutagenicity assays.

When appropriate, an oncogenic evaluation could be combined with a chronic feeding study.

B. Microbial Pest Control Agents.

1. Introduction. The Agency provides guidelines for the registration of four groups of microorganisms that may serve as pest control agents: bacteria, fungi, viruses, and protozoa. The Agency will develop guidance for testing other kinds of microbial agents (e.g., algae) as the need arises.

Hazard evaluations of microorganisms used as biological control agents are not on as firm a basis as is the case with conventional chemical control agents, simply because the field is relatively new and the interpretation of laboratory data in the light of mammalian hazard is difficult. Nevertheless, the Agency must use current methodology to assess the potential hazards associated with the use of microbial pest control agents. Much information is generated during the course of research and development of a microorganism as a microbial pest control agent, and industry is expected and, in many areas, required, to submit this information as part of the application for registration. The Agency looks to researchers in industry, academia, and Federal and State agricultural organizations for comment and information on the design and development of more relevant testing methodology so that specific issues of concern regarding the registration and use of the microbial agents can be addressed.

Accurate identification of the organism, as called for in § 151-20 of these guidelines, and its relationship to known human pathogens is essential before meaningful mammalian safety tests can be conducted and evaluated.

Moreover, information on stability, persistence, susceptibility to antibiotics, optimum conditions for growth *in vivo* and *in vitro*, recovery from host tissues and the natural environment, reliability of assay and test methods, resistance to chemical and physical factors, metabolic activities, knowledge of structural, biochemical, and other determinants involved in the infectivity-pathogenicity process, and certain other characteristics of the microorganisms are important as a basis for hazard evaluations. Information on the mode of action of a microorganism as an insect or plant pathogen can be analyzed and evaluated in terms of a possible identical or similar mode of action in mammals.

2. *Approach*. The safety evaluation must be developed for each group of microorganisms used as pesticides by taking into account the characteristics of the organism and its proposed use.

The tier approach is used to develop safety testing in a stepwise manner employing maximum challenges and proceeding, if necessary, with lesser challenges and other tests until the actual hazard of the agent can be quantified accurately.

The concept of maximum challenge experiments is useful when approaching safety testing of microbial agents because a candidate agent submitted for evaluation and testing is likely to be either totally innocuous to nontarget organisms or to be an opportunistic pathogen. It is clear that certain microbes known to be mammalian or human pathogens most probably will not be seriously considered for commercial development and will never reach the point of development requiring extensive safety tests. If the organism proves totally innocuous by the maximum challenge tests, further tests would be unnecessary. If a potentially adverse effect is detected in maximum challenge experiments, the tests proceed through the tiers until the actual hazard of the agent can be clearly evaluated.

Several important general properties of microbial agents have relevance for predicting human hazard. These include the ability to mutate, to form different virulence factors such as toxins and enzymes, and to otherwise change their spectrum of pathogenicity. Information on the ability of the organism to survive and replicate at mammalian body temperature is important to allow an evaluation of potential mammalian pathogenicity. Host range or species specificity information, if it exists, is important in assessing the hazard of the agent for mammals. It is also particularly useful in the evaluation of safety tests if methods for recovery of the organism in the presence of other similar organisms have been developed or are available.

In the development of these guidelines, it was recognized that no specific protocols could be provided at this time for the isolation and enumeration of the various microorganisms. These protocols will differ depending on the genus and species under test. In time, it is anticipated that specific protocols can be developed and then adopted by mutual consent of expert microbiologists for various genera of microorganisms that have proven useful as pest control agents.

3. Considerations for animal studies. The following discussion illustrates a number of important aspects of animal testing with microbial agents that should be considered by prospective registrants. Comments on these aspects are invited so that the Agency can determine their importance and relevance to the tests. In the conduct of animal tests, as much information as is feasible should be obtained in each experiment so that the data provided will permit well-considered judgments to be made regarding the hazards of use of the microorganism. For example, in LD50 tests information on gross pathology, dissemination, replication, and survival of the microorganism in animal tissues, organs, and fluids should also be obtained. Clinical signs of illness such as elevated temperature, unkempt appearance, altered feeding habits, weight loss, various forms of distress, depression, and other similar effects are also important observations, even though the animal may recover completely from the exposure. Animal excreta should be examined for the presence of the microorganism. An adequate number of animals should be exposed to the microbial control agent so that periodic sacrifices can be made to examine tissues and organs for gross pathology and the presence of the control agent. The organs or tissues receiving the initial challenge dose should receive close examinations for pathological changes, i.e., the lungs and upper respiratory tract for animals exposed to the aerosol, the intestinal tract for animals receiving the oral doses, and the skin of animals tested by the dermal route. Qualitative and quantitative measurements should be made to obtain evidence for survival and multiplication of the microorganism.

To study the infectivity of microbial agents, the urine and feces of test animals may be collected and examined for the presence of the inoculated microorganism. If present, serial passage of recovered isolates should be carried out with a standard serial passage procedure. The infectivity characteristics of the serially-passed isolates should be compared to the infectivity characteristics of the original test strain. Enhancement of virulence, survival, invasiveness, toxicity, or resistance to clearance mechanisms are indications of increased infectivity.

Overt symptoms of infection and disease related to pathogenic effects of challenging organisms will be considered as presumptively grave effects if observed in Tier I testing. However, the Agency recognizes that simple organism survival of the challenging organism is not generally regarded as a potential adverse effect, and that some survival over short time periods is an expected biological phenomenon. Studies have demonstrated that the mammalian system challenged with a non-proliferating organism will require some reasonable time period to cleanse itself of the challenge dose. (This time period is termed "period of clearance".) The period of clearance will vary, depending on the nature of the organism, the numbers in the challenge dose, the site of administration, and the immunocompetence of the animal. Survival of the test organism at the site of administration or at distant sites following administration for a period of only a few days will be considered as a negative finding.

The Agency considers that attempts should be made to establish mammalian LD50 values for all potential routes of human exposure, namely oral, dermal, and inhalation. Preliminary probing types of experiments would be conducted initially; that is, a few animals, with suitable controls, would be exposed to maximum feasible concentrations of the product. If no animals succumb, obviously no LD50 estimate is possible and no further testing is required. If sufficient animals show a lethal response, then appropriate stepwise dilutions should be tested to ascertain the LD50 value.

Dissemination rate, recovery rate, cloud concentration, and respiratory rate are the principal factors needed to calculate the dose administered by the respiratory route. Data from simple dynamic aerosol chambers that permit studies on whole-body or nasal-area-exposed animals may not yield this information unless the disseminatory device is capable of creating aerosols in the 5 micron range (pesticide particle size). A small aerosol size is necessary to assure that the microorganism reaches the alveolar spaces thereby providing the maximum potential for illness or death. Larger particles (microorganisms) may not reach the deeper recesses of the lungs as they usually lodge in the upper respiratory tract and are more readily cleared. It is also important that recovery data on microorganism viability both before and after dissemination from the device be provided so that doses can be estimated.

Appropriate controls must be tested to ensure that any illness or lethality is due to the presence of the active ingredient in the formulated product. Suitable controls for products containing replicable organisms would be "killed" preparations. Insofar as possible, the control for the test material should be identical to the formulated product except that it is biologically inactive. The manufacturer is generally best able to provide such material for the study.

In animals challenged by any of the various routes, an assay for antibody production should be performed by the most appropriate method that takes into consideration the animal species and the microbial agent involved in the test. This requirement necessitates pre-bleeding of the animals for base-line titers and additional bleedings for serum collection at the appropriate time following animal exposure. Such antibody tests may screen animals for previous exposure to the challenge organism and also demonstrate the ability of the animal to produce antibodies in response to the challenge dose. This test may also reflect the survival and replication of the microorganism in the host.

Since microorganisms and their spores are composed of protein, protein complexed with other biochemicals, and other antigenically active materials, it is very likely that hypersensitivity reactions of the non-immediate type will be achieved in experimental animals. This indicates the need for data on the development of hypersensitivity of the non-immediate type in animals; in these studies the technical control agent preparation is tested.

The Agency would also examine information on any hypersensitivity or allergic reactions that were experienced by personnel involved in the research or development of the biological control agent. The ease with which hypersensitivity reactions are achieved and the severity of these reactions in experimental animals should provide a basis, in conjunction with the reports from industry regarding hypersensitivity experience in workers, for evaluating the hazard of hypersensitivity for humans and, possibly, other mammals, associated with the use of the biological pest control agents.

4. Major concerns. (a) General. As previously stated, the major concerns of the Agency regarding hazards of use of replicating biological pest control agents affecting humans and other mammalian species are infectivity, allergenicity, irritancy, and toxicity. All candidate agents for registration must be tested for potential hazards. The amount of testing varies, however, for different types of agents based, in part, on the historical record of related organisms that showed a potential for hazard.

(1) Infectivity. The evaluation of potential microbial infectivity in mammals is based on information on the nature of the microbial agent and on data derived from studies especially designed to assess this specific hazard. In spite of the special design of infectivity tests in experimental animals, there is still some uncertainty in extrapolating results from experimental animals to man. The use of maximum challenge tests in a selected variety of test animal species is used to provide an adequate degree of confidence in the data. Much of the data characterizing the microorganism with respect to species specificity, infection processes, hosts and substrates, susceptibility to chemical and physical agents, and other similar factors, aids in evaluating the hazards of using the agent for pest control.

Section series 152 covers the submission of information on survival, distribution, replication, and gross pathology in test mammals following administration of the agent at high dose levels by various routes.

(2) Allergic responses. There is a definite possibility that a microbial pest control agent might induce allergic responses in nontarget animals and humans; this conclusion is based on the well-documented allergic responses elicited by the cell components of fungi, bacteria, and protozoa. Although it is possible that a virus could induce such a response, it is more likely that other constituents of the viral technical control agent formulation

would serve as allergens. In order to recommend adequate precautions in handling the material, its allergenicity potential must be investigated in test animals. In addition, information is also needed regarding any observations of skin sensitivity, respiratory or other allergic symptoms in people handling the material during development, production, and application.

(3) Irritancy. The potential for dermal and ocular irritancy, both of the technical and of the end-use formulated product, needs to be assessed as part of the primary battery of safety testing in order to adequately protect people handling and using the product. The following studies are presently considered sufficient to characterize this potential:

- (1) Primary skin irritation tests in laboratory animals by use of a patch test to intact and abraded skin, and, in certain instances, tests of irritation of the eye; and

- (2) Observations in exposed people during laboratory testing, pilot production, handling, transport, and field trials of the product.

(4) Toxicity. The toxicity potential may be associated with microbes which produce toxins related to their mode of action in target organisms, or metabolic products unrelated to their mode of action as biological pest control agents. Moreover, since products used as biological pesticides will be produced using fermentation technology or whole animal technology, very pure preparations of active ingredient (agent) in the form of the technical product are not expected. To test for the possibility of toxic components other than the replicating agent in relatively impure preparations, as well as to characterize the human toxicity potential of preparations which contain known toxic components for target organisms, it is necessary to perform, minimally, several acute toxicity studies on both the technical and end-use formulated products. These acute studies include acute oral and dermal tests and skin and eye primary irritation tests that are located in the Subdivision F guidelines.

(5) Immune responses. The Agency has provided guidelines for testing and data submission concerning the possible influence of biological pesticides on immune responsiveness and other immune parameters. Many assays are available to assess the effects of these agents on immunity.

Well-established immunological techniques are available that can provide information as to whether a substance affects the immune response. A wide variety of antimetabolites such as protein- and nucleic acid-inhibiting substances, x-irradiation, and corticosteroids, markedly suppress antibody formation in vitro. Such in vitro tests often correlate well with in vivo results. For example, almost all agents which suppress the antibodyforming ability of lymphoid cells in vitro have similar effects in vivo. It appears likely that these tests may provide some information on the carcinogenic activity of these agents, particularly with regard to effects on lymphoid cells.

(b) Specific concerns. (1) Bacteria. If, in the infectivity studies, a bacterial agent shows evidence of survival and replication in the test animal, the possible increased infectivity of the microorganism would obviously be of concern. Serial passage studies would be the next logical testing step so that new isolates could be compared to the original strain for enhancement of virulence, survival, invasiveness, toxicity, or resistance to clearance mechanisms. If such studies indicate poor genetic stability, further animal studies would be indicated.

(2) Fungi. As with bacteria, virulence enhancement is also a concern with fungi. Although relatively few fungi cause infections in humans with any regularity, the ability of certain fungi to adapt to the environment and grow under unfavorable conditions creates concern for the safety of humans and other mammals that may come in contact with fungi used as microbial pest control agents. Some fungi have a very wide range for growth, although the optimum temperature for a typical non-pathogen is generally much lower than the body temperature of mammals.

The degree of specificity shown by entomogenous fungi is quite variable. Some can grow only in insects, whereas others also can utilize substrates found in the soil and artificial media. Some fungi that grow on plants need nutrients supplied by the plant in order for the fungal spores to germinate.

Great diversity is often shown by fungi with respect to hosts, growth temperature, spore germination requirements, enzyme composition, resistance to physical and chemical conditions, nutrient requirements, dose necessary for infection and several other factors. Because of this diversity, a full characterization of the fungus, as outlined by §§ 151-20, 22, and -26, aids in assessing the hazards of use of the fungus as a microbial pest control agent.

The hazards of use undoubtedly will vary from one fungal species to another.

(3) Viruses. The potential lack of genetic stability of viruses with respect to species specificity is of concern to the

Agency. To test the capacity of a virus to infect and interact with nontarget species, a series of tests are proposed involving tissue cultures of cells of both humans and nonhuman origin. The tests invlude observations on gross morphological changes (cytopathic effects), inhibition of cell division, bioassays of culture fluid, decay of input virus, and potential appearance of viral proteins and nucleic acid.

While there is no evidence to indicate that any currently registered viral pest control agents represent a hazard to humans, a critical review of the literature indicates deficiencies in test systems utilized, in the kinds of data collected, and in the verification of testing results, such that the issue of potential hazards has not been completely resolved.

In recent years, new technologies have been developed that allow for a more precise evaluation of the potential for genetic instability in viruses. For example, the potential for genetic change can be assessed in the laboratory by several techniques. Tissue culture cells of human and nonhuman origin provide a sensitive means for testing the capacity of a virus to infect and interact with non-target species. Results from these tests, along with acute high dose infectivity and replication studies and standardized tests on irritation, hypersensitivity, and cellular immune response, will provide an appropriate data base for assessing the potential capability of viruses used as pesticides to cause adverse effects in humans.

It is important to note that special precautions are necessary to ensure that formulated products contain the desired infectious viral agent and are not contaminated with additional entities that could pose a hazard to humans. It is likewise important that the virus that is tested, whether in the formulated product, in the technical control preparation, or in its purest infective form, be identical to the virus in the commercial product. Because viruses are infectious agents that are widely distributed in nature and will be derived from crude material such as insects or tissue culture, it is possible for the viral control agent to be contaminated with other agents. The virus should be fully characterized as prescribed in the section dealing with the product analysis of microbial pest control agents (§§ 151-20 through -27).

In the course of development of an entomopathogen as a microbial pest control agent, the research and development activities could provide knowledge of structural, biochemical, and other determinants involved in the infectivity-pathogenicity process. Information on the mode of action of the microorganism as an insect pathogen can be analyzed and evaluated in terms of its possibly identical or similar mode of action in mammals.

(4) Protozoa. These microorganisms present several concerns for mammalian safety because of their ability to survive and to invade a wide spectrum of hosts, frequently including alternate hosts.

Many protozoa are able to survive and multiply in hosts at mammalian body temperature. Further, some protozoa are able to penetrate the gut or the intestinal tract and various body tissues mammals. Most damage to the insect host is caused during the vegetative, shizogonial period of development when the protozoa destroy host tissues.

Protozoan infection in insects results from the ingestion of protozoa contaminating the insect's food. During the use of protozoa as microbial pest control agents, infection in humans could occur following ingestion, but also could occur by respiratory or dermal routes of exposure. For this reason, all of these routes are to be investigated in experimental animals. For instance, if there is any evidence of oral infectivity in the acute test, a more extensive feeding study should more clearly define the hazard.

Since protozoan products used as biological pesticides will be produced from infected insects or possibly from tissue cultures, relatively pure preparations of active ingredient (agent) in the form of the technical product are not expected. To check for the possibility of acutely toxic components in relatively impure preparations other than the replicating agent, it is necessary to perform, minimally, several acute toxicity studies on both the technical and formulated products, including acute oral and dermal tests, and skin and eye primary irritation tests as required in Subdivision F.

5. Literature review and discussion of potential hazards. A few species of each of the four types of microorganisms (bacteria, fungi, viruses, and protozoa) have been investigated extensively for use as microbial pest control agents, and EPA registrations or experimental use permits exist for each of the four types of agents. A brief discussion follows on the various types with emphasis on the major mammalian hazard concerns.

(a) Bacteria. Laboratory and commercial preparations of Bacillus thuringiensis have been subjected to numerous evaluations for possible toxicity in vertebrates since 1957. Summaries of the studies conducted to determine the hazard to humans of use of bacterial agents as insecticides have been published.

(b) Fungi. EPA sponsored an American Institute of Biological Sciences workshop in 1975 to elaborate principles and criteria for establishing the safety of fungal agents (AIBS, 1975). There are nearly 100,000 species of fungi, but perhaps less than 50 cause human infection with any regularity, and clearly not any of these would be considered for use as a pesticide. One cannot ignore, however, the ever-increasing list of obscure fungi of pathogenic potential, even though the numbers of cases attributed to any one species remains low. For example, within a period of only two decades, two insect-associated fungi have been recognized as pathogenic for man, viz. Entomophthora coronata, causing rhinophycomycosis, and Basidiobolus meristosporus, the etiological agent of subcutaneous phycomycosis (Greer, 1977). Both organisms are thought to enter the host by trauma and remain localized at the site of injury; i.e., neither has displayed any tendency to become disseminated systemically.

Even more clearly associated with trauma are the etiological agents of mycetoma, a condition that tends to remain localized, and appears unrelated to predisposing factors such as immunosuppressive therapy. The overwhelming majority of cases are caused by a relatively few species (Emmons et al., 1977), but it should be considered that any fungus having the capacity to grow at the temperature of the human subcutaneous tissue may have this pathogenic potential. Similarly, there should be consideration of the potential for an agent to cause subcutaneous cysts (Emmons et al., 1977) The agents of both mycetoma and cysts are naturally resident in the environment.

Another disease caused by a variety of soil saprophytes including fungi and preceded by trauma is corneal ulcer formation. This condition, however, appears to be a consequence of immunosuppressive therapy, for fungal ulcers of the eye were virtually unknown until ophthalmologists began treating wounds of the eye by topical application of corticosteroids (Rippon, 1974). Many of the organisms causing corneal ulcers cannot grow at 37°C or more, a characteristic that allows screening for such agents.

Another disease that exemplifies the enlarging spectrum of fungi capable of causing systemic disease in man is that of the brain abscess caused by Cladosporium bantianum (trichoides). This condition was unknown prior to 1952. The organism is found in man's environment, has a predilection for the central nervous system, and has an unknown mode of infection. The condition does not appear to be linked to immunosuppressive therapy.

Testing for the pathogenic potentials described above is not feasible in every instance. There is no suitable experimental model for reproducing diseases such as mycetoma, cyst, and subcutaneous phycomycosis. There are, however, experimental systems for recognition of agents capable of causing systemic disease, especially those whose otherwise unexpressed pathogenicity could be potentiated by immunosuppressives; these are included in the scheme of testing described below.

Mycotoxicosis is a term used to broadly define toxic reaction due to ingestion of otherwise innocuous fungi or the metabolites of such fungi. Ergot and mushroom poisoning are examples known since antiquity. In the early 1960's, aflatoxins were discovered, first from Aspergillus flavus, but now from other species. Aflatoxins are acutely toxic to many vertebrates. At least one is carcinogenic, highly organ-specific, and causes liver tumors. There are now known to be more than 100 mycotoxins; thus, there is no single in vitro test that can be used for detection of all of them. Chemical analysis by chromatography has been widely used [thin-layer (e.g., Durackova et al., 1976); high-pressure liquid (Engstrom et. al., 1977)]. Enzyme-linked immunosorbent and immunocytochemical techniques have been tried (Lawellin et al., 1977), but biological assays, (i.e., feeding), remain indispensable. (For a recent bibliography, see Maggon et al., 1977).

Perhaps a more important aspect of pathogenicity of the fungi than either infectivity or toxicity is that of hypersensitivity reactions. These occur mainly as respiratory problems, categorized as either "immediate" or "nonimmediate" on an immunologic basis. Those of the immediate mechanisms are of the atopic variety exemplified by rhinitis and asthma; and those of the nonimmediate are exemplified by allergic alveolitis (hypersensitivity pneumonitis). These two major groups of hypersensitivity diseases are discussed below. A third disease entity, which is closely related to the second hypersensitivity category, is allergic bronchopulmonary aspergillosis, a common cause of pulmonary eosinophilia among atopics in Great Britain. The most common offending organism is Aspergillus fumigatus. Because of the known pathogenicity (including infectivity) of many species of aspergilli, it is doubtful that any would ever be under consideration for use as a pesticide.

Respiratory atopic allergies are a major health problem in this nation and elsewhere. They are incited by many substances other than fungi, but are commonly caused by fungi such as Cladosporium, Helminthosporium, Alternaria, Curvularia, Pencillium, and Fusarium.

Since most fungi have the capacity to cause sensitization reactions under ordinary circumstances, testing the capability of a fungal pesticide to sensitize would not provide any additional information of value. Information, however, is needed concerning the relative importance of an agent to elicit acute respiratory symptoms among the population at risk.

Allergic alveolitis, essentially a hypersensitivity pneumonitis, is presently the subject of intensive investigations in a number of institutions. This disease (which often provides descriptive clinical names such as bird fancier's lung, farmer's lung, cotton worker's lung) can be caused by a variety of materials including actinomycetes and fungi. One common feature of the disease, irrespective of the agent, is a history of long and repeated exposure to aerosols of the offending agent. The precise mechanism is not clearly understood, but allergy of the non-immediate type is involved, and patients usually have precipitating antibodies. The propensity of any dust, including fungal spore dust, to cause hypersensitivity can be determined in experimental animals by exposure to aerosols, or by inoculation intranasally or intratracheally.

Exposure of test animals to aerosols of mycelial fragments or spores of fungi has the advantage of approximating natural exposures. In these test systems, however, it is difficult to administer a predetermined dose deep into the alveoli and to confirm the actual dose delivered. Intranasal inoculation suffers from the same disadvantages. Intratracheal administration by cannulation is an alternative route of administration. Depending upon the animal species, cannulation is mechanically simple, and delivery of dose is reliable. Evidence of sensitization could be obtained by any of several standard procedures. Refer to Bice et al. (1979) for an evaluation of methods for pulmonary immunization.

(c) Viruses. More than 700 species of insects and several species of mites are reported to have viral diseases. Several of these viruses are registered as pesticides with the Agency and, to date, have not posed a threat to the health of humans or other mammals. These insect viruses are considered to be host-specific, most of them infecting only one host species or one insect group. They usually reduce insect populations only when a certain density of the insect population has been achieved, although the responsiveness to host density may be also dependent on the simultaneous occurrence of other environmental and host conditions.

However, most viruses are able to produce latent infections which, after stress, may suddenly manifest themselves as epizootics. Although it is assumed that viruses are species-specific, it is documented that viruses are capable of undergoing genetic change through a variety of mechanisms, many of which are not well understood. These mechanisms enable viruses to undergo genetic interactions with each other and to incorporate genetic material into the genomes of their hosts.

Because of the potential of viruses serving as biological control agents, a great deal of research already has been conducted. Most of the viruses affecting insects are either cytoplasmic polyhedrosis viruses or baculoviruses such as nuclear polyhedrosis viruses or granulosis viruses.

(d) Protozoa. Although many pathogenic protozoan parasites of insects exist, and some, such as gregarines and coccidia, may hold promise for use as insect control agents, most efforts to date have focused on microsporida. The genera Nosema and Vavria have received special attention, although scattered information is available for microsporida in other genera. Several points were considered in the development of the guidelines for evaluating the human hazards posed by these organisms. First, these parasites are widely distributed in nature, and large numbers are frequently encountered in foodstuffs and water. Second, some, such as Nosema algerae and Nosema locustae, have been the subject of numerous laboratory investigations and large scale propagation without any evidence of human disease. Third, serologic surveys of selected human populations specifically designed to detect antibodies to a variety of insect microsporidan parasites have failed to reveal any evidence of human infection (Chapupsky et al., 1972).

On the other hand, the protocols for evaluation of human hazards posed by the use of insect microsporida must take into account the following facts:

- (1) At least one microsporidian parasite affects a wide variety of animals;
- (2) Classification of the organisms is at present an uncertain and confusing issue; and
- (3) A few reports of human infections with microsporida do exist.

These facts are discussed briefly in the next few paragraphs.

Encephalitozoon cuniculi is the cause of subclinical granulomatous meningoencephalitis and nephritis in rabbits, mice, rats, and guinea pigs (Shaddock and Pakoo, 1971). It causes fatal encephalitis in carnivores such as puppies (Shaddock et. al. 1978), blue foxes (Nordstoga and Westbye, 1976), and some species of wild cats (Vavra et al., 1971), and the parasite has been reported several times in nonhuman primates (Anver et al., 1972; Brown et. al., 1973; and Siebold and Fussell, 1973). E. cuniculi produces more severe disease in immunologically compromised animals (Bismanis, 1970). Classification of the genera of microsporida is still an uncertain and confusing issue (Weiser, 1976). For about 10 years, E. cuniculi was classified within the genus Nosema (Lainson et. al., 1964). Encephalitozoon and Nosema are clearly different in several important features (Pakes et. al., 1975), yet the 10-year inclusion of these organisms in the same genus continues to raise some concern about the potential role of Nosema as human pathogens. The issue is not resolved, however, since one-way serological cross-reactivities between E. cuniculi and two species of Glugea as well as Nosema algerae have been shown recently (Nierderkorn, 1980). A few reports of human infection with microsporida exist. Several reports were incorrect (Barker, 1974) or have been retracted, but at least three cases have been documented (Ashton and Wirasinha, 1973; Margileth, et. al., 1973; and Matsubayashi et. al., 1959). The best documented case occurred in a young immunoincompetent child who succumbed with a variety of lesions including wide-spread microsporidosis (Lainson et. al., 1964). The organism was named Nosema connori (Shaddock and Geroulo, 1979) but its source was unknown. Indirect evidence that entomopathogenic Nosema may infect man includes the fact that Nosema algerae (a mosquito pathogen) can replicate (though poorly) in vitro in pig kidney cells at temperatures as high as 35°C, but not at 37°C. A few replicative forms of N. algerae have been found in subcutaneous tissues following injection of spores into the tail skin of mice. Mice injected subcutaneously in the tail produce antibodies.

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VI. RESIDUE ANALYSIS

A. Biochemical Pest Control Agents.

1. Background. A pesticide may not be used on a food or feed crop, or may not be employed for a use which may reasonably be expected to result (directly or indirectly) in residues in food or feed unless a tolerance or an exemption from the requirement of a tolerance has been established by the Agency, as provided for under Sections 406, 408, or 409 of the Federal Food, Drug, and Cosmetic Act (FFDCA) (21 U.S.C. 346, 346a and 348). The residue chemistry guidelines for tolerances/exemption are outlined in Subdivison O of the guidelines. The procedural regulations for filing petitions for a tolerance or an exemption from a tolerance are included in 40 CFR 180.7.

2. Approach. The full set of residue chemistry guidelines outlined in Subdivision O may not always be applicable to biochemical pest control agents for the following reasons:

- (1) Biochemical agents occur naturally in the environment or are identical to naturally occurring biochemicals and have properties similar to their natural counterparts; and

- (2) Many biochemical agents are used at very low application rates [i.e., 0.7 ounces (20 grams) active ingredient or less per acre]; and

- (3) Past experience indicates that biochemical agents are relatively nontoxic. Consequently, the resulting residues of biochemicals in food or feed would be very low and the potential for adverse effects would be correspondingly low.

Thus, the Agency believes that significant human dietary exposure will generally not occur from the use of biochemicals.

3. Tier Progression. In general, when no potentially adverse effects are observed during the Tier I toxicity testing (§§ 152-10 through -18), a biochemical would be exempted from the need for a tolerance, providing it is applied at rates of 0.7 ounces (20 grams) or less per acre per application. In this situation, the Agency would waive the usual metabolism and residue data and would recommend that an exemption from the requirement of a tolerance be made.

The full range of residue chemistry data as detailed in Subdivision O would apply to:

- (1) All biochemical agents proceeding to toxicity testing beyond Tier I (that is, to Tier II or III, as described in §§ 152-19 through -29 of this subdivision); and
- (2) All biochemical agents to be applied on food or feed crops at a rate greater than 0.7 ounces (20 grams) active ingredient per acre per application.

These would include plant metabolism studies, residue data, and analytical methodology. In addition, depending on the level of residue found in animal feed, the Agency may solicit data on animal metabolism and feeding studies to determine the carryover of residues into meat, milk, poultry, and eggs. When appropriate, tolerances for the latter commodities would be necessary.

B. Microbial Pest Control Agents.

1. Approach. As with a biochemical agent, the use of a microbial pest control agent on food, feed, or raw agricultural commodities requires that a tolerance, or an exemption from the requirement for a tolerance, be established by the Agency. In considering exemptions from the requirement for tolerances, the Agency recognizes that these agents do not necessarily pose the same potential hazards as conventional chemical pesticides. In fact, certain characteristics of many of these agents suggest that they pose relatively less hazard. These characteristics are listed below:

- (1) The efficacy of the agent often depends upon its ability to replicate in the target pest which is not likely to remain on the crop after harvest.
- (2) The living form of the agent in most instances will usually not replicate in the absence of the specific target pest (e.g., insect host).

- (3) Certain environmental conditions such as sunlight, rainfall, winds, humidity, and temperature often greatly reduce the viability of the agent and, therefore, the residues of living organisms are apt to be small or relatively insignificant shortly after application.

- (4) When evaluated by the tier testing scheme, data supporting currently registered microbial agents indicate that microbial pest control agents would not likely pose a hazard to humans or other mammals.

- (5) In many instances where and when a microorganism is used as a microbial pest control agent, the microorganism is already normally present in the environment and has demonstrated no adverse effects.

- (6) Residues of microorganisms used as microbial pest control agents that are capable of replication on food or feed - a very remote possibility - will possibly be rendered nonviable or be removed by the usual processing of such foods and feeds (i.e., washing, drying, heat sterilization, and additions of sugar, salt, and other preservatives).

2. Tier Progression. The Agency evaluates residue data for microbial pest control agents used on food, feed, or raw agricultural commodities only if toxic or other harmful properties were observed in the maximum hazard toxicology tests (Tier I) prescribed in §§ 152-30 through -39 of this subdivision. If Tier I toxicology tests indicate no toxic or other harmful properties, then no residue data would be indicated and thus a recommendation for an exemption from the requirements of a tolerance can be made.

3. Major Issues. In many cases, a natural population of microbial agent may be present at some background level at the site where a microbial pest control agent is applied. It may therefore be impossible to distinguish between natural and introduced microbial populations and therefore be very difficult to establish and enforce tolerances for naturally occurring microbial agents. The Agency invites comment concerning the testing methods for establishment and enforcement of tolerances for naturally occurring microbial agents.

VII. NONTARGET ORGANISM HAZARD

The purpose of nontarget organism testing is to develop data necessary to assess potential hazard of biorational pesticides to terrestrial wildlife, aquatic animals, plants, and beneficial insects.

A. Biochemical Agents.

A-1. Terrestrial Wildlife and Aquatic Animals (General).

1. Approach. The Agency bases hazard evaluation of biochemical agents on tests similiar to those required to support registration of conventional chemical pesticides. However, the Agency is proposing to reduce the number of Tier I tests and to modify test designs. Tier I tests would be designed to determine whether LC50 or LD50 values are above a specified maximum test concentration or dose rather than to require that the LC_{50} or LD_{50} be determined. This modified test design would apply if death was not observed at the maximum concentration or dose.

The Agency believes that the reduced number of tests and the modified test designs are appropriate because:

- (1) Innate toxicity is not inherent to the nature and mode of action of biochemical agents;

- (2) Experience indicates that most of these pesticides will be applied at very low rates compared to conventional chemical pesticides, thereby reducing likelihood of significant exposure to nontarget organisms; and

- (3) Past experience indicates that most biochemical pest control agents are not acutely toxic; e.g., LC_{50} and LD_{50} values for most biochemical agents are greater than 5000 ppm or 2000 mg/kg, respectively (in avian species).

Both terrestrial wildlife and aquatic animal testing schemes use some or all of the exposure criteria -- low use rate, low exposure formulation, nonaquatic use site, and high volatility -- to screen out those pesticides that qualify for reduced testing.

Volatility and its use as a criterion are explained as follows. Volatility is a function of the vapor pressure of the biochemical and its ability to adsorb or absorb to a substrate, e.g., suspended sediment, feed, or soil. The Agency proposes that an estimate of volatility be derived from the ratio of the substance's vapor pressure and solubility in water. These data (vapor pressure and solubility in water) are outlined in the Product Analysis portion (section series 151) of this subdivision. For the purposes of these guidelines, a biochemical pesticide is considered to have high volatility if the estimated volatility (H) is greater than $5x10^{-5}$ atm. m^3/mole. The rationale for this criterion is presented in part VIII of this Discussion.

A more exact determination of volatility of the formulated product is outlined as a Tier II test (see § 155-4), and may depend upon the pesticide use site and adverse effects observed in Tier I tests. Additional considerations concerning the exposure criteria are explained later in this Discussion, as they apply to each testing scheme.

In general, biochemical agents control behavior, growth, and/or development of target organisms. Ideally, Tier I tests should be capable of detecting adverse effects resulting from the primary mode of action by which the pesticide would likely act on the nontarget organism. At the present time, however, the Agency does not believe that it is appropriate to locate behavior and reproduction tests in Tier I for two reasons. First, no obvious approach or battery of behavior tests are universally suitable for pesticide screening (Weiss and Laties, 1979). This is apparently a reflection of the youth of this scientific discipline. Second, there are no widely accepted short-term screening tests that indicate chronic growth effects and/or developmental effects from pesticides on terrestrial vertebrates.

The Agency provides the following criteria for determining need for testing of biochemical pest control agents beyond the first tier:

- (1) If signs of abnormal behavior are reported in Tier I tests at levels equal to or less than the maximum expected environmental concentration; or

- (2) If detrimental growth, developmental, or reproductive effects can be expected, based on:

  - (a) Tier I test data;

  - (b) Available fate data from the product's research and development;

  - (c) Use pattern information;

  - (d) Results of mammalian testing required in the Toxicology section series 152; and

  - (e) The phylogenetic similarity between target pest and nontarget organism; or

- (3) If the maximum expected environmental concentration is equal to or greater than 1/5 the LC50 values established in

  Tier I terrestrial wildlife studies, or equal to or greater than 1/10 the LC50 or EC50 values established in Tier I aquatic animal studies.

In addition, both Tier I and Tier II tests would be indicated if:

- (1) The pesticide is to be applied directly to water; or

- (2) High use rates are proposed; and

- (3) The biochemical agents are not volatile.

A-2. Terrestrial Wildlife.

1. Approach. Most biochemical agents will be applied in a manner that will result in exposure to terrestrial wildlife. Even biochemical agents that are identical to naturally occurring semiochemicals and hormones, when applied as pesticides, result in exposure to terrestrial wildlife which would be different (e.g., in terms of route, time, and/or amount) from that which would occur under natural conditions. The Agency, however, realizes that most biochemical agents are applied at very low rates, and also, many possess physiochemical properties (e.g., high volatility) that reduce exposure to terrestrial wildlife. On this basis (i.e., low potential for significant exposure) the Agency permits a reduction in both number of tests and number of organisms per test to support registration of biochemical agents. Testing guidelines for biochemical agents are, therefore, reduced from those in Subdivision E of the guidelines for conventional chemical pesticides.

Certain use patterns and formulations will greatly reduce exposure of birds and mammals to biochemical agents (e.g., confined traps, tabs nailed to trees, tree injections, and uses around buildings). Biochemicals used in this manner would be confined to very limited areas, unlike sprayed chemicals that contaminate a variety of wildlife habitats when broadcast over wide areas. Therefore, these use patterns and formulations could reduce exposure of terrestrial animals to biochemical agents to a point where further reductions in testing may be appropriate. The Agency invites comment on specific use patterns and formulations which would qualify for reduced testing.

2. Tier Progression. (a) Tier I and progression to Tier II. Under these guidelines, two tests on birds normally are indicated for all biochemical pesticides that are not highly volatile: an avian single-dose oral toxicity test (§ 154-6) and an avian dietary toxicity test (§ 154-7). (See § 154-1, Figure 1.) For biochemicals that are highly volatile, only an avian single dose oral toxicity test is indicated. The Agency is following this approach because it believes that dietary exposure of a highly volatile biochemical agent would not provide data useful for hazard assessment, since the biochemical agent would be quickly lost from the feed.

Sections 154-6 and -7 will provide adequate data on toxic effects, including abnormal behavioral effects, of biochemical pesticides to avian wildlife. One control group and one treatment group per test may be all that are necessary to provide satisfactory data showing no adverse effects. These tests are similar to §§ 711 and -2 in Subdivision E of the guidelines.

Toxicity data for human safety, section series 152, in most cases would be sufficent to assess potential effects to wild mammals. Tier II tests are indicated if any of the following occur:

- (1) Maximum estimated environmental concentration (maximum EEC) is greater than or equal to 1/5 the avian single-dose oral LD50 value converted to ppm or is greater than or equal to 1/5 the avian dietary LC50 value;
- (2) Signs of abnormal behavior are observed in the avian single-dose oral or the avian dietary toxicity tests at levels equal to or less than the maximum EEC;
- (3) Growth, development, or reproductive effects may be expected based on observed effects in the avian dietary toxicity test, available fate data, use pattern information, and results of tests required to support human safety, section series 152.

If none of the criteria above is met, additional testing at higher tiers (II through V) is ordinarily not indicated.

(b) Tier II and Progression to Tier III. No additional testing at higher tiers would ordinarily be indicated if:

- (1) Environmental fate characteristics indicate that the estimated environmental concentration of the biochemical pesticide in the terrestrial environment is less than 1/5 the avian dietary LC50 or the avian LD50 converted to ppm; and
- (2) The pesticide or any of its metabolites or degradation products are not stable in the environment and potentially toxic amounts are not likely to persist in avian feed.

Testing as outlined in Subdivision E of the guidelines would be the next step (Tier III, § 154-12) if:

- (1) Environmental fate characteristics indicate that estimated concentration of the biochemical pesticide in the terrestrial environment is equal to or greater than 1/5 the avian dietary LC50 or the avian singledose oral LD50 converted to ppm; or
- (2) The pesticide or any of its metabolites or degradation products are stable in the environment to the extent that potentially toxic amounts may persist in avian feed.

3. Major Issues. In the process of developing testing guidelines for terrestrial animals, the Agency recognized at least two areas that require outside input and comment. A discussion of these issues follows.

(a) Maximum test concentrations and doses. The Agency believes that satisfactory data can be generated from the avian single-dose oral toxicity test (§ 154-6) and the avian dietary toxicity test (§ 154-7) where only one concurrent control group and one treatment group are tested, if a maximum test concentration or dose is tested. Negative results from such tests would provide a high degree of confidence that no adverse effects are likely to occur from the actual use of the biochemical pesticide. If, however, effects are observed at these maximum test levels, then further testing at lower levels would be indicated in order to establish precise LC50 and LD50 values and corresponding 95 percent confidence limits.

Since most chemical pesticides are applied at rates measured in pounds active ingredient (AI) per acre (or in kilograms/hectare), and biochemical pesticides are usually applied at much lower rates [often measured in grams (ounces) AI per acre], an appropriate maximum concentration or dose should reflect the lower application rates. Therefore, the Agency has reduced the maximum testing concentration (5000 ppm) and dose (2000 mg/kg) established in Subdivision E for avian toxicity tests on chemical pesticides. The established levels are multiplied by the ratio of the proposed application rate in grams to the number of grams in one pound (454 g). The general equations are as follows:

$$\text{Maximum Test Dose} = \frac{\text{maximum application rate in grams AI per acre*}}{\text{454g**}} \quad \text{x} \quad \text{2000 mg/kg}$$

$$\text{Maximum Test Concentration} = \frac{\text{maximum application rate in grams AI per acre*}}{\text{454g**}} \quad \text{x} \quad \text{5000 ppm}$$

For example, a 20 gram/acre (49 gram/hectare) rate would give an 88 mg/kg maximum test dose and a maximum testing concentration of 220 ppm.

(b) Categorization of semiochemicals by structure/activity relationships. Categorization of chemicals is a method through which scientists can infer which chemicals present risks of harm to humans and to the environment (Slesin and Sandler, 1978). One categorization scheme that may be useful for determining hazard to terrestial animals from semiochemicals used as pesticides (e.g., pheromones) is based on structure/activity relationships. The study of structure/activity relationships seeks to find association between a substance's physical and chemical properties and its effect on biological activity (Slesin and Sandler, 1978). Recently McLeese et al., (1979) used structure/activity relationship in the assessment of adverse effects of some industrial chemicals to nontarget organisms, such as shrimp and clams. Since most semiochemicals used as pesticides are applied at very low rates and possess special physicochemical properties (such as high volatility) that lessen their exposure to terrestrial animals, acute toxicity data on terrestrial animals for each new semiochemical submitted for registration may not be necessary. Rather, a determination of hazard could be based on existing acute toxicity data for structurally similar semiochemcals.

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* For biochemical pest control agents.

** A typical application rate for conventional pesticides.

These guidelines for biochemical pesticides do not include use of structure/activity relationships. However, the Agency believes that this concept, when developed for semiochemicals, could provide an acceptable data base for hazard assessments concerning both terrestrial and aquatic animals. At the same time, it could reduce the data required for registering semiochemicals used as pesticides.

A-3. Aquatic Animals.

1. Approach. Many biochemical agents possess special physicochemical properties, or are applied in a manner such that they are unlikely to enter the aquatic environment in significant quantities. Consequently, they would not usually be expected to pose hazards to aquatic animals. It is on this basis (i.e., low potential for significant exposure) that the Agency reduces aquatic animal data guidelines for certain biochemical agents from those for conventional chemical pesticides in Subdivision E. Guidelines are reduced in two ways:

- (1) The fish acute bioassay test indicates that only one fish species, rather than two, be tested; and
- (2) One control group and one treatment group per test may be all that are necessary to provide satisfactory data showing no adverse effects.

Based on past experience with biochemical pesticides, the Agency believes that most will qualify for reduced testing. But, in any case, each biochemical screened out for reduced testing would have several criteria supporting such a course of action. They are:

- (1) Non-aquatic use; and

- (2) Low potential for significant exposure, based on use pattern, formulation, and/or

- (3) Low use rates, and/or

- (4) High volatility.

A discussion of these exposure criteria and their use follows.

A biochemical agent must meet the first criterion plus one or more of the last three criteria in order to qualify for reduced testing. A biochemical agent that meets only one of the last three criteria (low exposure use pattern/formulation, low use rates, or high volatility) might be considered a weak candidate for reduced testing, while one that meets two or three criteria would be a much stronger candidate.

(a) Non-aquatic use site. This criterion identifies those biochemical agents that are applied on land when used as directed. Biochemical agents that meet this criterion (are applied on land) qualify for reduced testing. Aquatic animals will be exposed when a biochemical is applied directly to water (e.g., mosquito larvicides and aquatic herbicides that are biochemicals), and the Agency believes that reduced testing for these biochemical agents is not warranted.

(b) Low exposure via use pattern or formulation. Use patterns or formulations such as confined traps, tree injections, hollow fibers, tape dispensers, and drip irrigation, greatly reduce possibility of aquatic exposure. Biochemical agents applied in one of these manners would qualify for reduced testing. There may be other use patterns or formulations that would largely preclude aquatic exposure. Also, there are many borderline situations that may warrant reduced testing, such as minor uses, single applications, ground applications, or soil incorporation. Consultation with the Agency may be necessary to evaluate these borderline situations on an individual basis.

(c) Low use rate. The Agency provides low use rate as a criterion for reduced testing on the premise that application of small quantities of biochemical in a terrestrial use pattern limits the amount of material available to reach water and reduces the likelihood that concentrations will be high enough to reach a hazard level. Biochemical agents applied on land at 0.7 ounces (20 grams) active ingredient or less per acre per application would qualify for reduced testing.

An alternative approach would be to define low rates in relation to the biochemical's natural (ambient) concentration in the environment. For example, if application of the biochemical increases its concentration to no more than two times the ambient concentration, then the rate used would be considered low and the biochemical would qualify for reduced testing.

(d) High volatility. High volatility is a physical characteristic of some biochemicals that, for terrestrial use patterns, would almost preclude potential for aquatic exposure. An insect pheromone, whose efficacy often relies on vapor phase contact by the target insect, is an example of a biochemical that meets this criterion. For these guidelines, biochemical agents with an estimated volatility (H) greater than 5×10^{-5} atm. m^3/mole would qualify for reduced testing. Such biochemicals would have a volatilization half-life of less than one day, and therefore would not persist in water.

2. Tier progression. Biochemical pesticides determined to have low potential for aquatic exposure would qualify for reduced testing but would still be required to have one freshwater fish acute bioassay (§ 154-8) and one aquatic invertebrate acute bioassay (§ 154-9) to support registration (see § 154-1, Figure 2). Protocols for these tests are similar to §§ 72-1 and -2 of Subdivision E. The Agency recognizes that these tests are designed to assess acute toxicity, and may not be entirely suitable when other modes of action are concerned. Nevertheless, the Agency believes that, until a more appropriate screening test is available, these bioassays, in combination with the screening criteria for reduced testing, provide adequate evidence as to whether aquatic exposure, if any, will be biologically significant.

Tier II Environmental fate tests (§§ 155-4 through -13) would be indicated if any one or more of the following occur:

- (1) Signs of abnormal behavior are observed in Tier I tests at concentrations equal to or less than the maximum expected concentration in water; or

- (2) The maximum expected concentration in water is equal to or greater than 0.1 of any EC_{50} or LC_{50} determined in testing required by §§ 154-8 or -9; or

- (3) Maximum expected concentration in water is equal to or greater than 0.01 of any EC50 or LC50 determined in testing required by §§ 154-8 or -9; and

- (4) Adverse effects on growth, development, or reproduction may be expected based on Tier I test data, available fate data (e.g., from the product's research and development), use pattern information, or available effects data on phylogenetically similar target species.

If none of the above criteria are met, then additional testing at higher tiers is not indicated.

Biochemical agents that have terrestrial use patterns but do not meet any of the other three criteria for low exposure potential may still not pose a significant hazard to aquatic organisms. But, at present, there are not sufficient data on the effects of biochemical pesticides to warrant fully reduced testing for these materials. Instead, an intermediate course of testing is provided, including both Tier I (aquatic animal tests, §§ 154-8 and -9) and Tier II (environmental fate tests, §§ 155-4 through -15) to support registration. Results of Tier I and Tier II tests would be evaluated, and any further testing, if needed, would proceed along the tier system in Subdivision E of the guidelines.

Biochemical pesticides applied to water do not qualify for reduced testing because exposure of nontarget aquatic fauna is unavoidable in such situations. The effects tests specified by the tier testing system in Subdivision E (§§ 72-1 through -6) and environmental fate testing (Tier II, Subdivision M) to support registration apply to these pesticides. Progression to Tier II testing applies only to biochemical agents screened out for reduced testing at Tier I, since Tier II testing automatically applies for all other biochemical agents. The data for Tier II are described in the Environmental Fate sections (§§ 155-4 through 13) of these guidelines.

If environmental fate characteristics indicate that the estimated environmental concentration of the biochemical agent in the aquatic environment is equal to or greater than 0.01 of any EC_{50} or LC_{50} determined in testing in §§ 154-8 or -9, then testing as in Subdivision E of the guidelines would be indicated (Tier III, § 154-13). If the estimated environmental concentration is less than 0.01 of the above-described toxicity values, then no additional testing would be indicated.

3. Major Issue. Refer to part VII A-2.3.b. of this Discussion for a discussion that also applies to aquatic animals: categorization of semiochemicals by structure/activity relationships.

A-4. Nontarget Plants.

1. Approach. The plant testing scheme is based on the tier testing scheme found in Subdivision J of the registration guidelines (Hazard Evaluation: Nontarget Plants). Those guidelines and their accompanying discussion should be perused with respect to the plant tier testing scheme, the tests, the dose and other testing information and the tier progression criteria. Testing procedures for biochemical agents should be similar to those for other chemical pesticides with respect to phytotoxicity studies, and would therefore be subject to the same guidelines of Subdivision J.

2. Tier Progression. Progression to Tier III and further plant effects testing would depend on whether there are any adverse effects to desirable plants at the Tier I level, and whether there is possible movement by soil, water, or air from the intended site of application to nontarget areas as determined by selected tests of Tier II (Environmental Fate, Series 155; see § 154-1, Figure 3). In the vast majority of biochemical pesticides, such movement might occur, but at levels far below those which would have a detrimental effect as determined in the Tier I tests.

A-5. Nontarget Insects.

1. Terrestrial insects. (a) Approach. Development of baseline (first tier) tests for biochemical pesticides is difficult, for the following two reasons:

- (1) Effects of these biochemicals will often be long-term (e.g., effects on growth) rather than acute; this type of activity does not lend itself to short term testing; and
- (2) Effects on development or behavior, unlike mortality, may be difficult to quantify.

Due to these factors, and due to the fact that research in this area is in the early stages of development, there are no widely accepted, simple tests for evaluating biochemical effects on behavior and development of nontarget insects.

In view of the above, the Agency will not outline any specific type of Tier I testing for effects of biochemical pesticides on nontarget insects. Rather, the registrant should report any adverse effects on nontarget insects noted during efficacy testing, including effects such as:

- (1) Mortality or other adverse effects (e.g., behavorial modification) on insect predators or parasites of the target organism; and
- (2) Direct adverse effects on pollinators, or repellent effects on pollinators.

If no such effects are noted during efficacy testing, and in the absence of any other data indicating potential for adverse effects, no nontarget insect testing will be indicated. However, if adverse effects are noted and/or auxiliary data indicate a potential for adverse effects, then Tier II (Environmental Fate) testing will apply. If fate data do not indicate exposure, no further testing will be indicated. If fate testing indicates exposure, the registrant should consult with the Agency regarding further testing. Testing at that point would most likely be simulated or actual field testing, and would be directed at the problem identified during efficacy testing or indicated by the auxiliary data.

(b) Tier progression. (1) Tier I. As pointed out in the preceding discussion on "Approach", the Agency will not develop any baseline data requirements specifically to assess effects of biochemical pesticides on nontarget terrestrial insects. Rather, Tier I will consist of data (such as efficacy data) submitted by the registrant or made available from another source. If no data exist to indicate potential for adverse effects on nontarget insects, no testing will apply. (See § 154-1, Figures 4 and 5.)

(2) Tier II. The data for Tier II are described in environmental fate testing, section series 155 of these guidelines. Should environmental fate testing indicate no potential for nontarget insect exposure, no further testing would be indicated. Indication of exposure potential would lead to further testing.

(3) Tier III. The registrant will have to consult the Agency prior to testing at the Tier III level. Testing required at this point would most likely be simulated or actual field testing; the specific type of testing required will depend on the type of problem identified during efficacy testing or indicated by auxiliary data.

2. Aquatic Insects. (a) Approach. Testing should follow the scheme outlined in Aquatic Animal Testing Scheme for Biochemical Pesticides at § 154-9. Results of testing through this scheme will answer:

- (1) Does aquatic invertebrate bioassay indicate potential adverse effects?

- (2) Does environmental fate testing indicate potential for aquatic exposure?

If the answer to either of these questions is "no," no further testing is indicated. If the answer to both is "yes," then testing should continue according to Subdivisions E and L.

(b) Tier progression. As noted above, no specific tests have been developed exclusively for assessing the hazards of biochemical pesticides to nontarget aquatic insects. Rather, the tier "system" used here is constructed from testing systems developed in other parts of the registration guidelines. Testing should be conducted according to §154-9 of Aquatic Animal Tier Testing Scheme for Biochemical Pesticides. If bioassays indicate potential adverse effects on nontarget insects and Tier II (environmental fate) data indicate potential for aquatic exposure, testing should be conducted according to Subdivisions E and L. If one of these potentials is lacking, then no further testing will be indicated.

B. Microbial Pest Control Agents.

B-1. Terrestrial Wildlife and Aquatic Animals (General).

1. Approach. In designing Tier 1 tests, the Agency has attempted to balance two opposing philosophies concerning the hazards of microbial pest control agents. On the one hand, due to the relatively small existing data base pertaining to microbial pest control agents and their theoretical potential for causing environmental damage, the Agency would wish to conduct extensive testing, regardless of the pesticide's use pattern. This would be done in an effort to determine, as conclusively as possible, whether or not the pesticide's host spectrum includes nontarget terrestrial and aquatic organisms. The opposing philosophy argues that extensive testing in search of nontarget hosts is not justified because:

- (1) The pesticide organisms have existed in nature for thousands or millions of years without affecting the nontarget organisms with which we are concerned;

- (2) Rather extensive testing on a few microbial pest control agents (e.g., Bacillus thuringiensis and the nuclear polyhedrosis viruses) has demonstrated the safety of these types of pesticides;

- (3) The application of a microorganism does not necessarily increase that particular microbial population to a level any higher than would have occurred under natural conditions; and

- (4) The known hazards of the classical chemical pesticides are far greater than any hazards known to exist with microbial pest control agents.

After considering the two aforementioned philosophies, the Agency has concluded that at least some (minimum) test data on terrestrial and aquatic organisms should usually be evaluated, regardless of the pesticide's site of application (outdoor) and apparent potential for exposure. These minimum data would be necessary for the following reasons:

(1) When a microbe is applied as a pesticide, great numbers of the microbes are placed in the environment outside (apart from) its host, at a discrete point in time (day of application), and spread over all living and nonliving components of the target site, as well as adjacent areas (due to drift); hence, in terms of numbers of nontarget organisms exposed, the number of different species exposed, and the degree of exposure (number of microbes per nontarget organism), exposure would probably be greater than under natural conditions; and

(2) Data on toxic or pathogenic effects are essential for hazard assessment purposes when terrestrial or aquatic organism are very likely to be exposed to a microbial pest control agent, especially when no fate data will be required by the Agency in the first tier of testing.

Pathogenicity and toxicity are the major effects of concern regarding terrestrial and aquatic organisms. Therefore, the Agency has developed guidelines that will allow hazard assessment of possible pathogenicity and toxicity problems. In addition, the Agency desires a high level of confidence that no adverse environmental effects will result from actual use of microbial pest control agents. Toward this end, the guidelines in Tier I reflect a maximum hazard approach to testing as described earlier. Negative results from tests using this approach would provide a high degree of confidence that no adverse effects are likely to occur from the actual use of microbial pest control agents. Prior to the Agency's considering the registration of naturally occurring and strain-improved microbial agents, applicants would submit only Tier I data on nontarget organisms. However, both nontarget organism data (Tier I) and environmental expression data (Tier II) would be evaluated prior to considering genetically engineered microbial pest control agents for registration.

2. Major issues. (a) Maximum hazard dosage levels. Unlike environmental levels which generally decrease following application of chemical pesticides, the environmental levels of microbial pest control agents and any associated toxins may, at least temporarily, increase when the product is effective. Therefore, the maximum hazard dose for Tier I testing will be based on some safety factor times the maximum amount of active ingredient (microbial agent or its toxin) expected to be available to terrestrial and aquatic plants and animals in the environment. The target hosts (e.g., insects) are likely to contain the highest concentration of the microbial pest control agent that will be available to nontarget terrestrial wildlife and aquatic animals following a pesticide application. The maximum amount of microbial pest control agent (active ingredient) that one infected host can contain is called the host equivalent in these guidelines. Since the host insect can vary greatly in size, and the number of hosts that could be consumed by a known terrestrial or aquatic predator will also vary, largely depending upon its size, the host equivalent was adjusted by a weight-to-weight ratio of test animal to infected host organism. Therefore, the maximum hazard dose in these guidelines is equal to the host equivalent multiplied by the ratio of the weight of the test animal (e.g., fish or bird) to the weight of the infected host organism, (e.g., insect larva.) The route(s) of administration (e.g., oral, parenteral) and the size of the test organism(s) will largely determine whether the maximum hazard dose will be a multiple or a fraction of the adjusted host equivalent amount. Obviously, the maximum hazard dose must be an amount that is technically feasible to administer to the test organism(s) and therefore the Agency will be flexible in its assessment of whether a high enough dose was used in any given test.

In the case of microbial pest control agents applied to the soil to control soilborne diseases affecting plants, the soil may contain the highest concentration of the agent. The host equivalent concept would not be applicable in this situation since the host, most often, will be a microscopic propagule (spore, oospore, sclerotium or chlamydospore).

The Agency realizes that it would be very difficult to establish specific LC_{50}, ED_{50}, or LD_{50} values (e.g., LD50 = 1000 mg/kg) and 95 percent confidence limits for most microbial pest control agents, because test data are not likely to exhibit a log-probit dose-response relationship that is typical of chemical pesticides. Therefore, data that establishes that the LC_{50}, ED_{50}, or LD_{50} is greater than the maximum hazard dosage level (e.g., LD50 >1000 mg/kg) would often be adequate for the purposes of hazard assessment. In most cases, testing at one maximum hazard dosage level is expected to be sufficient to evaluate effects.

(b) Maximum hazard routes of administration. Various routes of administration are provided in these guidelines. There is a general belief in the Agency, however, that a parenteral route [e.g., intravenous (IV), intraperitoneal (IP)] would provide an acceptable maximum hazard exposure to terrestrial and aquatic animals in Tier I tests. While this route of administration is environmentally unrealistic, the Agency believes that negative test results from testing by IV or IP injection and using maximum hazard dosage levels would provide a high level of confidence that no adverse effects would occur from the actual use of the microbial pest control agent.

(c) Age of the test animals. The Agency considers that sufficient immunological and physiological differences exist between immature animals and mature animals to suggest that immature animals are potentially more susceptible to infection and possibly to the effects of any toxin produced by the microbial pest control agent. Therefore, the Agency has developed age guidelines for the test animals in Tier I tests, and recommends the use of immature animals in keeping with the maximum hazard approach to testing.

(d) Methods for detecting effects. Unlike toxicity tests where mortality can usually be determined by observation, infectivity tests often require sophisticated assessment methods for detecting sublethal pathogenic effects. Due to the extremely diverse nature of the active component in microbial pest control agents [e.g., natural toxins, acellular agents (viruses), prokaryotic cells (bacteria), eukaryotic cells (fungi, protozoans, most algae)], many different methods to detect each agent and assess infections have been developed. To assist applicants and registrants, the Agency is providing a scheme which will show some acceptable methods for detecting pathogenic effects for each type of microbial pest control agent (Table 1). This scheme is a generalization and is not completely reliable. However, it should cover a majority of the microbial agents and methods for detection.

(e) Viruses: the most challenging problems. Viruses represent the most challenging problems because they are parasites at the genetic level. Unlike other pathogens, they are acellular in their parasitic state and are able to insert their genomes (genes) directly into the host cell(s) with no intervening parasite membrane (Kawanishi, 1979). Very often , the diseases caused by viruses are preceded by long periods of latency. Viruses in latent states are often undetectable by microscopy or serological techniques. Some of the detection methods proposed above could detect viruses even when they are cell-associated in the form of nucleic acid molecules. The Agency is not aware of acceptable screening test(s) to determine if viruses in latent states will actually cause adverse effects later on. Further research is needed in this area.

Detection of a virus in a latent state ordinarily would be considered a noninfectious effect. Noninfectious effects have been reported in infectivity tests with other microorganisms. For example, Ignoffo (1973) reports that noninfectious bacteremia and the presence of bacteria in tissue of vertebrates following administration of heavy doses of *Bacillus moritai* and *B. thuringiensis* have been observed. At the present time, the Agency has decided to place infectivity as its primary concern. For the present, the decision to require effects, excluding toxicity, will be handled on a case-by-case basis.

(f) Test protocols. No standard widely accepted test protocols are available to evaluate the safety of microbial pest control agents to terrestrial and aquatic animals. In lieu of established test protocols, the Agency is providing tentative guidelines, based on past experience and on selected published references, which would aid in the development of generally accepted testing standards and protocols.

Table 1--METHODS FOR DETECTING EFFECTS OF MICROBIAL PEST CONTROL AGENTS IN SAFETY TESTS

	Microbial Pest Control Agents		
Method of Assessment	Microbial Toxin	Cellular Agents (Protozoa, Fungi, Bacteria)	Acellular Agent (virus)
Histopathology	O	X	X
Serology*	N.A.	O	X
Nucleic Acid Hybridization**	N.A.	N.A.	O

Key to Table symbols:

O: All members of this group detectable by this method

X: Not all infections by this group are detectable by this method

N.A.: Not Applicable

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*Radioimmunoassay, Enzyme-linked Immunosorbent Assay, Immunoperoxidase Assay, Immunofluorescence Assay.

**DNA:DNA and RNA:DNA Hybridization Techniques.

(Source: C.Y. Kawanishi, 1979)

B-2. Terrestrial Wildlife.

1. Approach. These guidelines indicate two tests on birds for all microbial pest control agents: an avian single-dose oral toxicity and pathogenicity test (§ 154-16) and an avian injection pathogenicity test (§ 154-17). The avian single-dose oral toxicity and pathogenicity test would provide data on any toxic effects to avian wildlife from exposure to the microorganism or any toxin it may produce. This test would also provide data on pathogenic effects following an acute oral exposure. The duration of the study would be about 30 days in order to provide time for incubation, infection, and manifestation of pathogenic effects from the microorganism. Also, gross necropsies are indicated, and any lesions would have to be characterized. This test is a modification of the avian single-dose oral LD_{50} study found at § 71-1 in Subdivision E of the guidelines.

The avian injection pathogenicity test would provide data on the pathogenic effects of the microbial pest control agent on birds following a parenteral exposure. The guidelines for the duration of the test and gross necropsies are similar to the avian single-dose oral toxicity and pathogenicity test. In addition, however, investigation of specific organs, organ systems, and the site of injection for multiplication of the microbial pest control agent, are indicated. Further, any observed pathogenic effects (e.g., lesions) lead to an assessment of cause and a description of the detection methods used in the assessment. A standard protocol for this test is not currently available, although Friend and Trainer (1974 a and b) have administered duck hepatitis to mallard ducks via two injection routes: intravenously and intraperitoneally.

One combined test would necessitate the administration of the microbial pest control agent via two different routes (e.g., oral and intravenous injection) to the same group of test birds. A standard protocol for this combined test is not currently available. The two routes of administration would expose the microbial pest control agent to two radically different environments in the bird: the gut and the blood.

2. Tier Progression. (a) Tier I. If no toxic or pathogenic effects are observed after exposing birds to the microbial pest control agent via two different routes of administration (oral and injection) at the maximum hazard dosage levels, then no further testing of birds would be indicated. If toxic or pathogenic effects are observed at the maximum hazard dosage levels, then Tier II environmental expression tests (§§ 155-18 through -20) would be indicated. (See § 154-1, Figure 6.)

Data on wild mammal toxicity and pathogenicity (§ 154-18) are indicated on a case-by-case basis when data indicate that there is considerable variation in the sensitivity of different mammalian species to the effects of a microbial pest control agent, and when wild mammals would be heavily exposed to the microbial pest control agent under normal use. However, the toxicity and pathogenicity data in section series 152 of this Subdivision for evaluating hazard to humans and domestic animals are normally adequate to indicate hazard to wild mammals. If no toxic or pathogenic effects are observed in tests on mammals, then no further testing of wild mammals would be follow. If any effects are observed in tests on wild mammals, then Tier II environmental expression testing (§§ 155-18 through 155-20) would be indicated.

Genetically engineered microbial pest control agents are treated differently from microbial pest control agents that are identical to naturally occurring microorganisms or that are improved strains of naturally occurring microorganisms. Since genetically engineered microorganisms do not pre-exist in the environment, data on effects on nontarget birds and mammals in Tier I (§§ 154-16 through -18) and data on survival in the environment in Tier II (§§ 155-18 through -20) are indicated in order to assess potential environmental hazards.

(b) Tier II. The data outlined in Tier II are described in environmental expression testing (§§ 155-18 through -20) of these guidelines. If the expression characteristics preclude exposure of the microbial pest control agent to nontarget birds and mammals, then no further testing of these animals would be indicated. If Tier II tests indicate that birds and mammals will be exposed to themicrobial pest control agent, then testing at Tier III would follow.

(c) Tier III. The types of effects reported in the Tier I tests would determine which Tier III test(s) would apply. If toxic effects are reported in Tier I tests, and Tier II tests indicate exposure, then the guidelines of § 71-2 of Subdivision E would apply (see § 154-24). In this case, futher testing (if needed) would proceed as in Subdivision E. If pathogenic effects are reported in Tier I tests at an amount equal to the adjusted host equivalent, or if chronic, carcinogenic or teratogenic effects are reported in tests in §§ 152-50, -51, and -53 for evaluating hazards to humans and domestic animals, then a long-term avian pathogenicity and reproduction test (§ 154-26) would apply. This test would provide data on pathogenic effects of the microbial pest control agent on birds during a sensitive period in their life, breeding and reproduction. It would also provide data on the effects of the microbial pest control agent on avian reproduction. This test would be a modification of the avian reproduction study (§ 71-4) in Subdivision E of the guidelines. If no pathogenic or reproductive effects are observed, the Agency would, at this time, review all the data and determine if decisions regarding registration can be made.

Pathogenic effects occurring at Tier III and beyond raise serious questions concerning the registration of any microbial pest control agent. Also, testing at Tier IV, simulated and actual field testing for mammals and birds (§ 154-33) may not be feasible, since it may not be possible to confine the microbial pest control agent to a test area and prevent it from escaping to contaminate adjacent areas. The applicant should seriously reconsider the proposed registration of any microbial pest control agent that requires Tier III or IV testing. If a decision cannot be made without further testing, and the microbial pest control agent can be restricted to a field test area, then testing at Tier IV would be indicated.

(d) Tier IV. Simulated and actual field testing (§ 154-33) would provide data on the pathogenic effects of the microbial pest control agent on birds and mammals following field applications at actual label use rates. This test would be indicated when pathogenic effects are reported in Tier III testing (§ 154-26) at levels equal to actual or expected field residue exposure levels, and when the Agency is reasonably confident that quarantine methods will prevent the microbial pest control agent from escaping from the test area to contaminate adjacent areas. The specific type of test (small-pen, large-pen, or full-scale field test) should be discussed with the Agency before beginning the study.

3. Major issues for discussion. In the process of developing the guidelines for terrestrial animals, the Agency recognized many important areas that require outside input and comment. The Agency needs scientific input and invites comments on the following issues of concern:

(a) In vivo testing. The guidelines outline *in vivo* testing of birds and mammals. *In vitro* testing may be considered in the future. Wolf (1975) has suggested a two-pronged testing approach for safety testing of baculoviruses, using both *in vivo* and tissue culture (TC) testing. He reported that there are established or permanent cell lines for duck embryo fibroblasts, chicken embryo fibroblasts, as well as representative mammalian cell lines from a bat, rabbit, mouse, and deer. Ignoffo (1973) reported that at least 12 viruses, including all major viral types, have been tested *in vitro* in either avian egg embryo fibroblasts (chicken or turkey), fish, or mammalian cell lines. Virus multiplication or cytopathic effects were reported for one nuclear polyhedrosis virus in chicken embryo cells and human amnion tissue, and for one noninclusion virus in chicken embryo cells and mouse sarcoma tissue. In contrast, no effects were observed *in vivo* when rabbits and mice were injected or fed the latter virus.

The Agency is not convinced at this time that the results of *in vitro* tests can be used exclusively to determine potential adverse effects to individual terrestrial animals (e.g., endangered species) or populations of terrestrial animals in the environment.

(b) *Test substance*. Microorganisms used as pesticides could be applied in any one of a combination of naturally existing forms. It is preferable that the test organism be exposed to the most infectious form, whenever infectivity is the primary hazard of concern. Similarly, when toxicity (e.g., a microbial toxin) is the hazard of concern, the test organism should be exposed to a form of the microbial agent in which the toxin would be most readily available. Unfortunately, there is no easy way to determine which is the most infectious or toxic form of the microorganism to the test organisms. The route of administration may also play an important role in determining which form should be tested. For example, if the route of administration is intravenous, then the active vegetative cells of a bacterium, or the infectious hemolymph may be more appropriate than vegetative cells or polyhedryde, respectively.

For these guidelines, testing the technical grade of the active ingredient applies in all tests except the simulated and actual field testing (§ 154-33), when the use of the formulated product applies in order to simulate or reproduce actual field use. This provides the consistency between the tier tests necessary for assessment of hazard.

(c) *Route of administration*. These guidelines outline testing by oral gavage and via injection by a parenteral route (preferably intravenous or intraperitoneal). It is important to note that the administration of test material to 10 to 17 day old birds by oral gavage will likely require the use of small needles or cannulae with ball-tipped ends in order to prevent injury to the birds. Ignoffo (1973) reported that the following groups of terrestrial animals have been tested *in vivo* for effects caused by entomopathogens:

Group	Routes of Administration
Mammals (primarily laboratory populations)	- diet, oral, inhalation, subcutaneous, dermal application, intradermal, intraperitoneal, intravenous, intracerebral, intranasal, intramuscular, eye application
Birds (chickens, and laboratory populations that are phenotypically similar to wild species)	- oral, diet, intraperitoneal (chickens)

At the present time there is no general consensus within the Agency concerning the route of administration that would consistently provide the maximum hazard exposure to nontarget birds or mammals. There is, however, a general belief that one of the parenteral injection routes may be appropriate. Also, since the gut normally provides such a radically different environment from that in the rest of the bird or mammal body, and since insectivorous birds and mammals can be expected to ingest large quantities of actively growing microorganisms when they feed on diseased insects, the Agency believes that the oral route would also be appropriate. The dietary route of administration was considered for Tier I tests, but the Agency beieves that it does not generally reflect the maximum hazard test philosophy for Tier I tests. The diet, however, is considered to be an appropriate route of administration for Tier III and Tier IV tests (§§ 154-26 and 154-33).

The Agency is aware of the theoretical potential of microbiological pesticides to disrupt the function of rumen bacteria. At present, the Agency is seeking further information concerning the possibility of such effects on wild mammals. If any such effects were to be reported in safety tests on domestic ruminants, then the Agency would solicit similar tests on wild ruminants.

The Agency recognizes that a combination of administrations in one test (e.g., oral and intravenous or intraperitoneal injection) may be possible. It would certainly be in keeping with the maximum hazard testing philosophy, and would reduce testing time and expense. However, combined exposures could unduly traumatize the test organisms so as to cause mortality, or in some other way cause spurious results.

(d) Avian test species. These guidelines provide that young bobwhite quail or mallard ducks be tested in Tier I tests. Birds between 10 and 17 days of age at the beginning of the test period should be used in the avian single-dose oral toxicity and pathogenicity test and in the avian injection pathogenicity test. Within a given test, all birds should be the same age. (U.S. Environmental Protection Agency, 1978).

Summers et al. (1975) suggest testing two species of birds including at least one insectivorous species. Wolf (1975) stresses that test organisms should represent insectivorous and herbivorous species. He suggests testing blackbirds, yellow-billed cuckoos, representative members of the swallow family, and ducklings.

In Subdivision E of the guidelines, the Agency suggests bobwhite quail, ringneck pheasants, and mallard ducks as acceptable test species for avian acute toxicity tests of chemical pesticides.

The following facts influenced the Agency's proposal to test bobwhite quail and mallard ducks in avian toxicity and pathogenicity tests of microbial pest control agents:

- (1) These species are ecologically significant and widely distributed in the United States;

- (2) They have proven to be good laboratory test species and are appropriate for acute, subacute, and chronic testing;

- (3) Laboratory populations are genotypically and phenotypically comparable to wild species;

- (4) There is a large body of data on the effects of chemical pesticides on these species available for comparison purposes;

- (5) It has not been determined if any avian species or group of avian species is a better indicator of potential effects from microbial pesticides than bobwhite quail or mallard ducks; and

- (6) Testing species from the family Icteridae (e.g., blackbirds, grackles, and cowbirds) may be ecologically significant and in line with the maximum hazard philosophy, but would present many practical problems in rearing, reproduction, controls, and handling.

In support of testing immature birds in Tier I, the Agency notes that insects are vital to immature birds during the first two or three weeks of life, and make up a much larger proportion of their diet during this time than at other times in their life. Thus, they are functionally insectivorous birds at this age. Also, for the purposes of pathogenicity testing, the Agency feels that sufficient immunological and physiological differences exist between immature birds and adult birds to warrant considering the immature bird as potentially more susceptible to infective challenge, and so proposes their use in the maximum hazard testing approach.

(e) Selection of dose levels. For Tier I tests, the Agency suggests that a maximum hazard dosage be administered. For the acute oral test for toxicity and pathogenicity, the maximum hazard acute oral dose should be no less than 10x the adjusted host equivalent amount. [The adjusted host equivalent is equal to the host equivalent times the ratio of the weight of the test animals to the weight of the host organism. The host equivalent is equal to the maximum amount of active ingredient that one infected host (e.g., a late instar larva) can contain.] If the calculated amount of microorganism is determined to be excessive, a 5x or 2x amount may be used. If the microbial pest control agent produces any toxic or pathogenic effects at the maximum hazard dose level, then testing at lower doses would be indicated. If the microbial agent kills test organisms at the the highest dose level, then sufficient doses and test organisms would be required to determine an LD50 value, if possible. For the injection pathogenicity tests, the maximum hazard dose level should not be less than one adjusted host equivalent. If this amount is not feasible, a 1/2x, 1/5x, or 1/10x amount could be tested if a rationale is provided to support the reduction. If the microbial pest control agent produces any pathogenic effects at the highest dose level tested, then testing at lower doses would be indicated.

It has been suggested (Ignoffo, 1973, and Summers, 1975) that microbiological pesticides be tested at 10x to 100x the average field dose per acre with a conversion ratio of the weight of the test animals to the weight of a 70-kilogram man. This may be appropriate for safety tests required for human safety, but the Agency believes a different approach may need to be taken for safety tests to provide data on effects to wild birds and mammals. The Agency has information that one infected host organism can contain a quantity of active infectious agent greater than the amount applied to one acre. Considering that diseased host insects are likely to lose their natural defense mechanisms and become easy prey for birds and mammals, and that birds and mammals can consume more than one infected host in a day, a maximum hazard dosage should be greater than the amount of microbial pest control agent in one infected host. Since the number of infected hosts that a bird or mammal can consume is a factor of the size of the predator and the size of the infected host, the host equivalent can be adjusted by the factor equal to the ratio of the weight equivalent can be adjusted by the factor equal to the ratio of the weight of the test animal to the weight of the host organism:

$$\text{adjusted host equivalent} = \text{host equivalent} \times \frac{\text{weight of test animal}}{\text{weight of host organism}}$$

An added safety factor (e.g., 2x, 5x or 10x) could be applied to the adjusted host equivalent. Of course, if the calculated amount is not feasible, then the safety factor may be reduced. For injection pathogenicity tests, it may be necessary to reduce the adjusted host equivalent by some factor such as 1/5x, 1/2x, or 1/10x. All modifications to the adjusted host equivalent dosage amount which would be determined by the applicant should be supported by a rationale based on sound scientific reasoning.

(f) Length of tests. The guidelines provide that the duration of all Tier I tests be about 30 days long. This should permit time for incubation, infection, and manifestation of effects in the test organisms for most microbial pest control agents.

Various authors have proposed test duration times for toxicity and pathogenicity tests ranging from 14 to 35 days (Ignoffo, 1973; Ignoffo et al., 1975; Summers, 1975). The Agency realizes that the test duration period may be unnecessarily long, or may not be long enough to detect effects such as viral diseases that recur after prolonged intervals of latency, e.g., *Herpes zoster* (Fenner et al., 1974). At the present time, however, the Agency is not aware of an accurate method to predict whether a virus detected in a test organism will manifest latent effects. The Agency invites comments on the proposed test duration period and the probability of encountering microbial pest control agents with latent effects.

(g) Synergism with chemical pesticides. The Agency is aware of the synergistic effects of some chemical pesticides. Where information is available, the Agency uses it to determine potential hazards from combinations of chemical pesticides in formulations and from sequential applications of different chemical pesticides. At the present time, the Agency does not regularly require applicants to test for synergistic effects from chemical pesticide combinations.

Successful pest control has been achieved with some combinations of microbiological and chemical pesticides. They offer the benefits of immediate pest reduction plus long-term control. Friend and Trainer (1974 a and b) reported findings that suggest synergistic response in mallard ducks to the combined effects of different organochlorine pesticides and duck hepatitis virus (DHV) when compared to the effects of the different organochlorine pesticides (DDT and Dieldrin) and DHV alone. A similar interaction could potentially occur with microbial pest control agents that exhibit effects in Tier I testing and chemical pesticides.

The Agency believes it would be inappropriate to impose a Tier I requirement to test for synergism on microbiological pesticides at this time. However, if data suggest that a microbial pest control agent and a chemical pesticide will act synergistically, and the microbial pest control agent and the chemical pesticide will be combined in one product for use outdoors, or if the labeling of the microbial agent recommends combination use with the chemical pesticide, then the Agency may request Tier I tests on the formulated product in addition to Tier I tests on the technical grade of each active ingredient. For all such combinations, the Agency will determine the need for additional data on a case-by-case basis.

(h) Synergism with other biorational pesticides. Combinations of different microbial pest control agents as well as combinations of a microbial pest control agent and a biochemical pesticide (e.g., pheromone) are being tested for use in a single formulated product. Preliminary tests have shown, in some cases, additive and synergistic effects on the target organism(s). Although the Agency knows of no instances where these combinations have resulted in additive or synergistic effects on nontarget organisms, such effects are a possibility. As with the previous issue [(g) above], the Agency feels that it would be inappropriate to impose a requirement to test for synergism on microbial pesticides at this time. However, if any data suggest that a particular biorational pesticide, when combined in a product for use outdoors, will act synergistically, then the Agency may request Tier I tests on the formulated product in addition to Tier I tests on the technical grade of the active ingredient.

(i) Protocols. Generally acceptable testing protocols are needed to complete and finalize these guidelines. In the interim, scientifically sound protocols are acceptable subject to prior review by the Agency.

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B-3. Aquatic Animals.

1. Approach. The Agency has considered several criteria that could be used to determine the extent of testing for effects on aquatic animals in Tier I. They are:

- (1) Site of application and resulting potential for aquatic exposure;
- (2) The natural geographic distribution of the microorganism;
- (3) The natural population level of the microorganism compared with population levels likely after application;
- (4) Ability of the microbial pest control agent to survive and replicate after application; and
- (5) The extent to which the microorganism has been manipulated or genetically engineered.

While all of the aforementioned criteria are important, the Agency has chosen site of application and its resulting potential for aquatic exposure as the key criterion for establishing the extent of initial effects testing for microbial pest control agents. The rationale for selecting this single criterion is that it direcly addresses the most critical issue regarding potential hazard: likelihood of exposure. Furthermore, criteria 2, 3, and 4 would be implicitly considered in connection with the criterion for site of application. Tier II (environmental expression) testing, would always apply in the case of genetically engineered microorganisms.

The Agency recognizes that considerable judgment will be required to properly employ site of application as a criterion. While many uses obviously entail direct application to water (e.g., mosquito control and aquatic weed control), the Agency also intends that less obvious or borderline uses also be subjected to the expanded testing. Some examples that fall into the latter category are applications to forests, drainage ditches, riverbanks, and partially aquatic crops such as rice. Widespread applications to major crops such as cotton, soybeans, and corn could also warrant expanded testing. To the extent possible, the Agency will rely on its experience with the classical chemical pesticides in distinguishing between terrestrial and aquatic use patterns in borderline situations.

2. Tier Progression. (a) Tier I. For microbial pest control agents applied in terrestrial use patterns (where direct aquatic exposure is not anticipated), one freshwater fish (§ 154-19) and one freshwater aquatic invertebrate (§ 154-20) should be tested to assess toxicity and pathogenicity. For microbial pest control agents applied directly to fresh, estuarine, or marine waters, one additional fish species and one additional invertebrate species should be tested in Tier I (see § 154-1, Figure 7). These tests should be conducted as 30-day static or static renewal bioassays using one or a combination of methods to administer the pesticide (e.g., aqueous, dietary, or injection). These tests should be designed to simultaneously assess both toxicity and pathogenicity as well as to detect and quantify the microbial agent in the test animal.

No further testing would be indicated if: (1) results of the aforementioned tests indicate no toxic or pathogenic effects, and (2) host spectrum or beneficial insect tests indicate that the microbial pest control agent has a narrow host spectrum such that crossover into nontarget aquatic invertebrates is not likely. If toxic or pathogenic effects are observed, then environmental expression testing (Tier II) would follow. If host spectrum or beneficial insect tests imply crossover into nontarget aquatic invertebrates, then additional aquatic invertebrate species (those expected to be susceptible) would have to be tested in Tier I, or as an alternative, Tier II testing would have to be conducted. If tests on additional species indicate toxic or pathogenic effects, then testing at Tier II would be indicated; if otherwise, then no further testing would be necessary.

Genetically engineered microbial pest control agents would be treated differently from microbial pest control agents that are identical to naturally occurring microorganisms or that are improved strains of naturally occurring microorganisms. Since genetically engineered microorganisms would not have existed previously in the environment, the more extensive data on effects to nontarget fish and aquatic invertebrates in Tier I (§§ 154-19 through -21) and data on survival in the environment in Tier II (§§ 155-18 through -20) are indicated in order to assess potential environmental hazards.

(b) Tier II. The data for Tier II are described in environmental expression testing sections (§§ 155-18 through -20) of these guidelines. If the environmental expression characteristics do not indicate exposure of the microbial pest control agent to nontarget fish or aquatic invertebrates, then no further testing of these animals would be indicated. If Tier II tests indicate that fish and aquatic invertebrates will be exposed to the microbial pest control agent, then testing at Tier III is indicated.

(c) Tier III. Whereas Tier I tests are designed to screen microbial pest control agents using a maximum hazard testing scheme, Tier III tests are intended to more precisely evaluate and quantify the actual hazard associated with the microbial pest control agent. The types of effects reported in Tier I tests would help determine which Tier III test(s) would be required. If only toxic effects are observed in Tier I tests, then the guidelines of §§ 72-1 through -6 of Subdivision E would apply, and further testing would proceed as in Subdivision E. If pathogenic effects or both pathogenic and toxic effects are observed in Tier I, then tests that could be indicated in Tier III are the following:

- (1) Additional acute or subacute test(s) of fish or aquatic invertebrates to evaluate the spectrum of susceptible nontarget species, or determine the susceptible route(s) of exposure, or determine the doseresponse relationship between the pesticidal agent and susceptible nontarget organism(s) (§ 154-27);
- (2) Fish embryolarvae and aquatic invertebrate life cycle studies (§ 154-28);
- (3) Aquatic ecosystem test(s) (§ 154-29);
- (4) Test(s) to evaluate the potential for opportunistic infections (§ 154-30); and
- (5) *In vitro* studies such as tissue culture (§ 154-30).

If results of Tier III tests indicate no pathogenic effects, then no further testing would be indicated. Conversely, if results of Tier III tests, along with environmental fate data, indicate toxic or pathogenic effects, then simulated or actual field testing (Tier IV) may be warranted.

(d) Tier IV. Simulated or actual field testing (§ 154-34) provides data on the pathogenic effects of the microbial pest control agent on fish and other aquatic animals following field applications at actual use rates. This test would apply when pathogenic effects are reported in Tier III testing (§§ 154-27 and -29) at levels equal to actual or expected field exposure levels, and when the Agency is reasonably confident that quarantine methods can confine the microbial pest control agent to the test area and prevent contamination of adjacent areas. The specific test would be determined on a case-by-case basis after consultation between the Agency and the registration applicant.

3. Major issues. This section identifies and discusses issues regarding aquatic testing of microbial agents that require outside input and comment. Most of the issues stem from two problems:

- (1) There are no standard widely accepted test protocols available to evaluate the effects of microbial pest control agents on nontarget aquatic animals; and

- (2) There are some potential hazards associated with the use of microbial pest control agents that the Agency recognizes and for which practical methods of evaluation are unavailable. The role of *in vitro* testing and Tier IV testing is also discussed in this section.

(a) Issues associated with Tier I protocol. The desired Tier I test protocol would simultaneously assess toxicity and pathogenicity in aquatic animals. The maximum hazard test philosophy would be exerted in terms of treatment level, method of pesticide administration, and age of the test animal.

A Tier I test should be conducted as a static or static renewal bioassay. The microorganisms should be administered:

- (1) As a suspension in the water (aqueous exposure);

- (2) In the diet in the form of diseased host insects or treated feed;

- (3) By injection; or

- (4) Preferably as a combination of all three routes of exposure.

If any test animals die during the test, the cause of death (e.g., toxicity, pathogenicity) should be determined, if possible. This information would be used to determine what further tests, if any, are warranted. Exposure and observation should extend for at least 30 days. Individual test animals animals should be removed periodically throughout the test period and at test termination for examination to assess pathogenicity.

If a sublethal infection is observed in test animals prior to test termination, it may be necessary to continue the observation period in order to more adequately assess the significance of the infection (e.g., will it be lethal?). Several published studies address certain aspects of the above-described protocol: Committee on Methods for Toxicity Tests with Aquatic Animals, 1975; Ignoffo et al., 1973; Van Essen and Anthony, 1976; Wolf, 1975; Lightner et al., 13 Couch et al., 1975; and Hetrick, et al. 1979.

The following paragraphs discuss, in more detail, some of the Tier I aquatic organism tests.

(1) Test organisms. The guidelines provide that the species tested be selected from the list of species recommended by the Committee on Methods for Toxicity Tests with Aquatic Organisms (p. 21) (1975), with the exception of goldfish. These species are desirable test organisms for several important reasons:

- (1) They are used to evaluate chemical pesticides, and therefore EPA has considerable background data on these species for comparative purposes;
- (2) Standard methods for the care and handling of these species are available; and
- (3) The species are widely distributed, are generally available, and have a variety of food habits and habitat requirements.

When possible, consideration should be given to testing species representatives of the geographic region where the microorganism is to be applied, and when applicable, species likely to prey upon or scavenge the diseased target host animals should be tested.

Unless there are other overriding considerations, the rainbow trout should be used as the freshwater fish test species. It is a desirable test animal because:

- (1) It is a good indicator species in terms of sensitivity to chemical toxicants;
- (2) It is partially insectivorous;
- (3) No other species has been shown to be preferable in terms of sensitivity to microbial pest control agents;
- (4) There is considerable background data on this species pertaining to its microbial diseases (Mann, 1978); and
- (5) Standard tissue culture procedures are available for this species (Wolf and Quimby, 1969 and 1973).

Use of young fish (3-6 months old) is preferable since they would be more likely to display a lethal pathogenic effect, whereas older fish may simply carry the infection and not die.

Due to the broad phylogenetic spectrum from which to choose, it is difficult to select the most appropriate aquatic invertebrate. Generally, a test organism that is phylogenetically similar to the target host should be chosen. Such a test organism would be the most likely to be susceptible to infection by the microbial agent. Therefore, when evaluating a microbial agent whose target host is an insect, it would be appropriate to choose an aquatic insect (e.g., caddisfly) as the nontarget aquatic invertebrate test species.

Daphnia, a Cladoceran, has the advantage of having considerable background data for comparative purposes. Pound (1977) exposed the entomopathogen *Mattesia* to *Daphnia* and observed a bioconcentration effect. This resulted from the filter feeding habits of *Daphnia*, and is a desirable feature in terms of assuring that the test animal ingests the microorganism. Both *Daphnia* and certain aquatic insects have the advantage of a short life cycle or aquatic phase, and both undergo periods of natural stress and potential susceptibility to the microorganism as a consequence of molting. A potential drawback of these species is their small size which would preclude the use of injection as a route of exposure, although insects as small as adult mosquitoes can be injected with as much as one microliter of solution. The use of crayfish or possibly a freshwater prawn would overcome this problem and retain the advantage of testing an animal that would molt during the test exposure period.

(2) *Method of pest control agent administration*. Three methods of pesticide administration should be considered:

- (1) Suspension in the test water (aqueous exposure);
- (2) Dietary, in the form of diseased target host animals or incorporated into the standard feed; and
- (3) Injection.

If appropriate, and when possible, all three routes should be used simultaneously in a single test to ensure that the susceptible route of exposure, if any, has been tested and to challenge the non-target test animal with the maximum possible hazard. Different pathogens may be capable of infection by different routes of exposure, so that no single route may adequately screen all microorganisms. Each of the proposed routes has certain advantages and disadvantages. Therefore, a multiple route of exposure would be extremely beneficial and cost-effective in screening microbial pest control agents.

Addition of the microorganism directly to the test water is a routine procedure. It simulates the type of natural exposure that could occur immediately after application of a microbial pest control agent. It also simulates the route of exposure by which many known pathogenic agents infect fish and aquatic invertebrates. However, care must be taken to assure that a high concentration of microorganisms (in the water) does not lower water quality to an unacceptable level.

Dietary exposure also simulates certain natural conditions. In fact, it is perhaps the most important means of infection for the normal hosts of entomopathogenic agents (Surtees, 1971). Therefore its use in evaluating effects on nontarget fish and aquatic invertebrates is logical. This route offers a further advantage: it increases the possibility of exposing the test animals to a different life stage of the microorganism than may be present in the formulated product if diseased target hosts (e.g., insects) are used as the feed.

An injection (e.g., intraperitoneal for fish) does not simulate a natural route of exposure but it does insure that a known quantity of microorganisms is brought into direct contact with the test animal at the cellular level. Also, for many microorganisms, injection would probably be the most stringent test for infectivity. This route may subject the test animals to some stress, but it has been used routinely to assess microorganism effects in aquatic animals. Protocols for the aqueous exposure, dietary, and injection methods of administration are described by the Committee on Methods for Toxicity Tests with Aquatic Organisms (1975); Van Essen and Anthony (1976); and Lightner et al. (1973), respectively. Undeen and Maddox (1973) also describe the injection method. Macek et al. (1979) describe studies in which fish were simultaneously administered chemical pesticide by aqueous and dietary exposure.

Finally, oral intubation is another possible route of exposure, and is one that has been used to evaluate microorganism effects in fish (Savan et al., 1979; and Narayanan et al., 1977). This route has the advantage of assuring that a known amount of test material is ingested. This advantage, however, does not outweigh the risk of injury or undue stress that could result from using this method in combination with an injection. Therefore, the oral intubation method is not recommended.

(3) Test substance. The substance to be tested will depend in part on the method of pesticide administration used in the study. It is essential to test the most challenging form of the microorganism (in terms of pathogenicity or toxicity). It is equally important to test the life stage(s) to which nontarget aquatic animals are most likely to be exposed. These objectives should be achievable through the use of multiple routes of administration, provided it is known which form is most challenging and which form is most likely to be encountered by the nontarget animal. The technical grade of the active ingredient (the microorganism as it exists in the formulated product) should be used for the aqueous exposure. The formulated product should be tested if it is to be applied directly to water. The material to be injected should be the form(s) or life stage(s) of the microorganism that best meets the two aforementioned obectives (i.e., most challenging form, and form to which nontarget animals are most likely to be exposed). The use of diseased target hosts would be most desirable for the dietary exposure. If this is not feasible, then treated feed should be prepared, using the same life stage(s) that appear in diseased target host organisms.

(4) Selection of treatment concentrations. Treatment concentrations must be related to the number of microorganisms to which aquatic animals may be exposed under actual use conditions. And, in

keeping with the maximum hazard philosophy, treatment concentrations must be relatively high. Consideration must be given to the level of exposure resulting from direct application as well as exposure resulting from consumption of diseased target host organisms (usually insects). Exposure, in terms of frequency and number of microorganisms, could be extremely high in the latter case.

Treatment concentrations for dietary exposure would be determined by the degree of infection in the diseased host. If possible, hosts killed by the pathogen should be used to assure high numbers of microorganisms, and the presence of any toxin that may have been formed. Treated feed should be fortified to contain at least 1x, and if feasible, 2-10x the host equivalent. (Host equivalent is the maximum amount of active ingredient that one infected host can contain.)

The highest feasible concentrations should be used in the aqueous exposure and injection. At a minimum, the concentration for aqueous exposure should equal the theoretical concentration present in six inches of water immediately after a direct application of the microbial pest control agent to six inches of water. A treatment concentration 100-1000x this level would be preferable to impose maximum hazard and incorporate an ample margin of safety. However, the use of such a high concentration may be limited by its adverse effect on water quality such as oxygen depletion and production of metabolic wastes by the microorganisms. The treatment concentration used for an injection should be linked to host equivalent. If possible, the injected material should contain a concentration of microorganisms equal to or greater than the adjusted host equivalent.

$$(\text{Adjusted host equivalent} = \text{host} \times \frac{\text{weight of test animal}}{\text{weight of host organism}})$$

In certain situations this concentration would not be feasible and would have to be reduced. Reduced concentrations should be a fraction of the adjusted host equivalent, in the range of 0.1-0.5x. Modifications to the adjusted host equivalent dosage amount should be supported by a rationale based on sound scientific reasoning.

(5) Test duration. Exposure and observation must be extended to at least 30 days (unless test animals die) to allow time for any potential infection, microorganism replication, or pathogenic or toxic effects to manifest themselves. If a sublethal infection is observed, then the test should be extended to evaluate the significance of the infection. Similarly, if test animals begin to die near the end of the 30-day period, the test should be continued to determine the fate of the remaining test population.

The 30-day test duration was selected on the basis of past research [Savan et al. (1979); Pound (1977); Van Essen and Anthony (1976); Lightner et al. (1973)]; and the recommendation of Summers et al. (1975). Certain factors may dictate that this period be modified. For example, if infection and death of target hosts is normally not evident for many days (i.e., 20-30), it would be logical to lengthen the period of exposure for the test animals. Conversely, a shorter period of exposure may be warranted in tests using animals with short life cycles (i.e., Daphnia or mysid shrimp).

(6) Observation and examination of test animals. Daily observations are required to record mortalities and note any behavioral, pathogenic, or toxic effects. Test organisms must be examined for infection or any microorganism-related effects periodically throughout the study and at test termination. The most difficult aspect of this requirement is the verification of the presence or absence of an infection. The general methods of assessment that may be required to make this determination include histopathology, serology, and nucleic acid hybridization. These methods, and the situations in which their use may be appropriate, were presented in the general discussion of terrestrial and aquatic animals, part B-1.(2)(d), and in Table 1 of this Discussion.

Undeen and Maddox (1973) used the following criteria in their work with Nosema algerae to distinguish between a true infection and microorganisms observed in the test animal. In a true infection:

- (1) Both vegetative forms and spores had to be present in the test animal; and
- (2) The number of spores recovered had to exceed the number injected by 100x.

This type of approach may be useful for certain other microorganisms.

(b) Tier III test protocols. The embryolarvae, lifecycle, and aquatic ecosystem tests in Tier III (§§ 154-28 through -29) would follow the same general protocols that are referenced for these types of tests in Subdivision E of the guidelines (§§ 77-4 through -7). However, generally accepted standard protocols for conducting these studies with microbial pest control agents have not been developed. In fact, few, if any, such tests have ever been conducted with microbial pest control agents. Therefore, at the outset, the Agency recognizes that new and different test designs and test parameters may be more appropriate than modified Subdivision E tests. Research and methods development are needed in this area before the Agency can make specific recommendations concerning protocols and tier progression.

(c) The role of in vitro and Tier IV testing. The Agency recognizes that there are in vitro tests available to assess the infectivity of certain microorganisms, one of which is tissue culture for viruses. Cell lines are established for several species of fish (Wolf, 1975), and such a test might be a useful means of assessing infectivity in certain situations. However, the relationship between effects demonstrated by *in vitro* tests and effects likely to occur under *in vivo* situations is uncertain. For example, Ignoffo (1975) states that "Tissue, completely non-susceptible in the intact organism, may support viral multiplication when explanted into a culture media." Therefore, the results obtained from tissue culture tests could be useless in accurately predicting environmental hazard. Another potential drawback of tissue culture studies is that, often, no host cell culture (e.g., insect cell culture) has been developed. Therefore, such a study would have no positive control group and the validity of a negative result would always be subject to some doubt.

The Agency has concluded that, at the present time, *in vitro* studies such as tissue culture cannot be substituted for the *in vivo* studies provided in Tier I. At the same time, the Agency recognizes the potential value of these studies for the following purposes:

- (1) As a relatively inexpensive and rapid means to screen for potential infectivity in a broad spectrum of species; and

- (2) As a test to support or check the results of *in vivo* tests.

Therefore, a provision for tissue culture studies is included in Tier III of the testing scheme.

(d) *Tier IV Testing*. The Agency recognizes the possible danger in using simulated or actual field tests (Tier IV) as the final test of the safety of a microbial pest control agent. If an agent has progressed through the tier system and requires a field test, it must have displayed significant adverse effects in some or all of the previously conducted laboratory tests. This fact would argue against the use of a field test, since such a test could release potentially hazardous microorganisms, with the potential to proliferate in the environment and cause widespread environmental damage, unless adequate quarantine measures could be taken. Therefore, before any Tier IV field test is to be undertaken, the applicant should discuss its plans with the Agency concerning potential hazards. If the Agency determines that a Tier IV field test would pose an unacceptable risk, then the microbial pest control agent would not likely be acceptable for registration.

On the other hand, the Agency also recognizes the potential value of Tier IV simulated or actual field tests as a further check on the safety of microbial pest control agents that demonstrate no hazard in Tier I tests, or that demonstrate a hazard that could be adequately controlled by quarantine methods in the field. These tests could be conducted concurrently with full scale efficacy testing, and the Agency would strongly encourage such testing. This would provide the opportunity to evaluate pesticidal effects (both direct and indirect) on a much broader spectrum of nontarget species than is possible in Tier I testing.

(e) Assessment of other potential hazards: opportunistic infections and latent viruses. Opportunistic infections in nontarget aquatic animals are recognized by the Agency to be a potential hazard. A similar concern is noted for the formation of latent viruses. Research indicates that aquatic animals may be rendered significantly more susceptible to microbial infection, (e.g., by viruses and bacteria) when stressed by such factors as Aroclor 1254 (Couch and Courtney, 1977), copper (Hetrick et al., 1979), temperature, salinity, pesticides, and other pollutants, (Snieszko, 1974, and Schwartz, 1974). This increased susceptibility raises several important questions:

- (1) What is the likelihood of an opportunistic infection (from a microbial pest control agent) occurring in an abnormal host such as a nontarget aquatic animal?

- (2) Will the proposed Tier I test adequately screen microbial pest control agents for potential opportunistic effects? Or could a microbial pest control agent be non-infective in a Tier I test, but infect stressed nontarget animals?

- (3) Will a latent virus be detected by a Tier I test and, if so, how can its significance be assessed?

There is far too little background information and research on microbial pest control agents to suggest an answer to the first question. However, the Agency believes that the potential for this type of problem should not be ignored. The Agency is confident that both sublethal infections and latent viruses produced in Tier I tests can be detected if the proper methods of detection are employed. However, the potential for an apparently non-infective agent (in Tier I testing) to infect stressed animals is unknown. And, at present, the Agency is not aware of any practical, generally accepted, routine screening test that could be used in Tier I to determine the potential for such an occurrence. If a sublethal infection is observed in Tier I, then further testing may be warranted. A microorganism/stress interaction test is proposed in Tier III as a means of assessing sublethal infections, but further research is needed to develop the protocol for such a test. With regard to latent viral infections, the Agency is not aware of a standard method to evaluate the potential for a latent virus to reactivate and cause adverse effects in aquatic animals. Further research is required.

(f) Oncogenic effects. The Agency recognizes the potential for oncogenic effects that are associated with viruses. The probability of oncogenicity in nontarget aquatic animals, as a result of exposure to a viral pesticide, is unknown. At this time, the Agency is unaware of any standard method that could be used to screen for such an effect. Further research is required to develop an appropriate test and determine when its use is justified.

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B-4. *Nontarget Plant Testing*.

1. *Approach*. The plant testing scheme proposed herein is based on the tier testing scheme found in Subdivision J of the registration

guidelines (Hazard Evaluation: Nontarget Plants). Those guidelines and their accompanying preamble should be perused with respect to the plant tier testing scheme, the tests, and the dose.

The Agency has determined that phytotoxicity data for conventional chemical and biorational biochemical pesticides will be requested only on a case-by-case basis. We have based this on the premises that these substances readily degrade, their long-term effects are minimal, and that most off-target effects are due to environmental and/or application equipment factors. On the other hand, the Agency has determined that phytotoxicity studies should be submitted for all microbial agent pesticides. The pesticide science for these agents is relatively new and their adverse effects can be far-reaching. These effects may occur because the microbe is capable of extensive regeneration in a favorable environment. If it is transported to other off-target areas and the selectivity is not known, considerable damage to desirable plants can occur.

2. Discussion and major issues. Plants and animals of an undisturbed natural system exist within a narrow balance. This balance is maintained by competition for space and by the biological control of those organisms that might become pests to the system. During a disturbance of the balance, a biotic entity for which natural enemies in that area do not exist could be introduced and become dominant. Such is the case with a number of plants that are now destructive weeds and such could be the case for introduced organisms that man may use to attack these weeds. However, sufficient surveillance and careful examination of such introduction can prevent the adverse effects to the extent that introduced weeds are causing.

Of great economic and natural threat to a majority of the aquatic areas of Florida and one which is spreading to aquatic areas from California to Virginia is the water hyacinth, Eichornia crassipes. A biological control agent has been found for this menace in Cerospora rodmanii, a fungus. It is highly specific, easily disseminated, and has not been found to affect man, wildlife, fish or domestic animals (Freeman, 1977). It is so specific that, in tests with over 80 higher terrestrial and aquatic plants, there was no infection of healthy plants (Conway and Freeman, 1977). Unhealthy plants may, however, be detrimentally affected. A request for an experimental use permit has been granted by the EPA for testing in Florida and Louisiana.

Algal blooms have destroyed once clear lakes and ponds throughout the United States because of introduced contamination. The various algal blooms are in many instances being partially controlled by agents such as viruses that are cultured and employed as microbial pest control agents. A number of the viruses have been identified along with their respective host(s) (Brown, 1972). One of the better known and first to be identified is LPP-1 which attacks species of Lyngbya, Plectonema, and Phormidium.

The primary concerns with respect to the use of living organisms on or near desirable or nontarget plants in the control of pest plants are the selectivity of the organisms, the purity of the organism or strain produced (quality control), and the persistence or lack thereof of the organism. Both Freeman (1977) and Brown (1972) stated several criteria that must be satisfied in varying degrees in order for the pathogen to be a desirable candidate as a biological control agent for pestiferous plant species. These criteria include:

- (1) Selectivity for the specific pest organism;
- (2) Absence of adverse effect on man;
- (3) Absence of adverse effect on domestic animals, fish, wildlife, and desirable insects;
- (4) Absence of adverse effect on nontarget or desirable plants;
- (6) Absence of any detrimental effects on water quality;
- (7) Lack of accumulation in non-target organisms;
- (8) Ease of production, dissemination, and self-maintaining when established;
- (9) Effectiveness under the environmental conditions of the intended use locations; and
- (10) Simplicity of assay for its presence in small amounts, both quantitatively and qualitatively.

With respect to selectivity, the most important consideration is that a number of strains of the pesticide organism may exist, each being selective for an individual or group of organisms. Therefore, when an organism is mass-produced and used in areas other than its original habitat, the organism must be tested for its selectivity not only to the specific pest plant but also to closely related (same genus or family) nontarget plants.

Testing procedures similar to those found in the Subdivision J guidelines, i.e., the testing sections for both the target areas (§ 121-1) and nontarget areas (§§ 122-1 through 125-4), would be used. (See § 154-1, Figure 8.) Plant species and varieties similar to the pest and to the desirable target area plants would be tested in addition to a basic set of plants for possible susceptibility.

During mass production of live organisms to be used for pest control, other strains may develop or chemical by-products may be introduced that are injurious to plants in general. Quality control testing is necessary to determine any detrimental effects of the end-products of the production process. This testing may be done by chemical analyses (primarily for toxic chemical by-products) and biological analyses for purity of the end-product. The biological analysis would have to be tailored to each organism to be tested but would follow the applicable guidelines as found in Subdivision J.

It may be desirable for the pesticide organism to have some persistence characteristics. Such characteristics would include ability to survive introduction and to grow and reproduce in sufficient numbers that the organism will be efficacious. Where persistence occurs due to sporulation, detrimental effects of the viable spores and their products would need to be determined. Testing for the persistence of the pesticide organism which might exist in the presence of nontarget plants and for any detrimental effects due to this quality would need to be conducted in a manner similar to the selectivity testing noted above.

In the testing regime, one of the considerations with respect to uses for pest animal control is whether the material is to be used within its area of natural occurrence. Where a microbial pest control agent is proposed for use in an area where it does not naturally occur, tests would be performed to determine if the microbial pest control agent is phytotoxic and, if so, the extent of the effect.

The ultimate test in all of these schemes would be the evaluation of these microbial control agents for nontarget plant resistance and susceptibility under field conditions. Conway and Freeman (1977) have stated that because many plants do not show defined responses to a pathogen, criteria are needed to evaluate the degree of damage necessary to classify a fungus (or other microorganism) used for biological control as a threat to other crops and nontarget plants. The data supplied as proposed by these guidelines should provide the necessary information to meet this criterion for the evaluation process.

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B-5. Nontarget Insects.

1. Terrestrial insects. (a) Approach. Assessment of potential nontarget insect hazard from uses of microbial pest control agents is made difficult by a number of factors:

- (1) Most microbial pest control agents will be specifically selected and/or designed for their ability to control pest insects. As such, nontarget insects represent the organism group most at risk, being, in most cases, relatively closely related to the target organism.

- (2) There are very few nontarget insects whose importance to man can be measured economically. There are many species whose importance (e.g., in a food chain, or in regulation of population levels of a crop pest) may be difficult to justify the expenditures necessary for testing and evaluation.

- (3) Unlike chemical pesticides, most microbials will work through pathogenicity rather than toxicity. The simple, short, first-tier tests which should suffice for hazard evaluation for some chemical pesticides will not be appropriate for microbial agents. Adequate assessment of pathogenicity will demand time to evaluate the microbial agent for infectivity and for its ability to reproduce or develop in the test insect.

- (4) Hazard evaluation for a microbial pest control agent will involve determination of the host range as an important factor. A problem here is that extrapolation, even across species lines, is often not dependable. For this reason, the Agency will provide for testing with representatives from a number of "beneficial insect" taxa. Information from these tests will be used in conjunction with host range data (developed during efficacy testing) to develop a clearer idea of the overall insect host range.

The Agency is aware that this first tier of testing may, in some cases, be more extensive than the baseline data requirements in Subdivision L. However, there should be very few microbials which require effects testing beyond the baseline level.

In view of the factors cited above, the tier-testing scheme for microbial pest control agents is based on a fairly extensive first tier. The purpose of the Tier I testing is to assess toxicity and pathogenicity of the microbial agent to the honey bee and to three species of predaceous and parasitic insects. Selection of the predator/parasite species to be tested should take into account such factors as the likelihood of exposure to the microbial agent, phylogenetic proximity of the test species to target pest species, and similar relationships. A rationale for selection should be developed by the registrant.

Testing beyond Tier I, will vary depending on whether or not the microbial pest control agent is a genetically-engineered microorganism. While further testing for non-genetically-engineered microorganisms will depend on results of Tier I testing, genetically-engineered microorganisms will automatically go to Tier II testing (environmental expression). Beyond this point, the testing for the two types of microbial pest control agents is substantially similar. The testing scheme is discussed in detail detail in the following sections on tier progression.

(b) Tier Progression. (1) Tier I. Under these guidelines, toxicity/ pathogenicity tests on the honey bee and on three species of insect predators and/or parasites are indicated for all microbial pest control agents (see § 154-1, Figures 9 and 10). Selection of predator and parasite species for testing is made by the registration applicant. Species selected should be representative of groups which will be exposed under the conditions of proposed use, and which have some important relationship with the target pest. Rationale for selection is to be provided by the registrant. The main purpose of the Tier I testing is to determine presence of toxic or pathogenic effects on representatives of a few major orders of beneficial insects. As noted above, the representative test species selected, in addition to the honey bee, should be of some importance in the agroecosystem to be exposed to the microbial control agent. Data derived from Tier I testing will be used in conjunction with available information on use pattern, host range (specificity), fate, and other similar factors, to assess potential for adverse effects. If data indicates no potential for adverse effects, no further testing would be indicated. Should the results of Tier I testing indicate toxic and/or pathogenic effects, then Tier II testing (environmental expression) would follow.

For all genetically-engineered microorganisms, testing includes appropriate Tier I tests and Tier II (environmental expression) testing.

(2) Tier II. The data for Tier II are described in environmental expression testing (§ § 155-15 through -23) of these guidelines. For nongenetically-engineered microorganisms, if expression characteristics preclude exposure, no further testing would be indicated. If data indicate that nontarget insects will be exposed to the microbial pest control agent, then the registration applicant should consult with the Agency regarding possible Tier III testing.

For all genetically-engineered microorganisms, available information on use pattern, host range, and other similar factors should be used in conjunction with the Tier I and Tier II data to assess the microbial agent's potential for adverse effects on nontarget insects. If no adverse potential is indicated at this point, no further testing would be indicated. If data indicate that the potential for hazard exists, data on expression characteristics should be closely examined to determine exposure potential. If expression characteristics do not indicate exposure, no further testing would be indicated. Determination that certain susceptible nontarget insects would be exposed should usually lead to consultation with the Agency regarding further testing or registrability of the product.

(3) Tier III. For all microbial pest control agents, Tier III consists of advanced tests specifically responding to adverse effects identified in earlier tier testing. Such tests may be simulated or actual field tests, but further research is needed to develop the protocols for such testing. In any case, Tier III testing would be preceded by consultation with the Agency.

2. Aquatic Insects. (a) Approach. Tier I testing, as outlined in the "Aquatic Animal Tier Testing Scheme for Microbial Pest Control Agents" (§ 154-1) will include toxicity/pathogenicity testing with Daphnia, or a species of aquatic insect, or both, depending on use pattern. Detection of pathogenicity/toxicity in Tier I testing will automatically lead to expanded testing which, if the impacted site is fresh water, will most likely involve testing with aquatic insects.

VIII. ENVIRONMENTAL FATE AND EXPRESSION

A. Biochemical Pest Control Agents: Environmental Fate Testing.

1. Scope and approach. The term environmental fate pertains to biochemical pest control agents (whereas the term environmental expression pertains to microbial pest control agents). The purpose of environmental fate testing is to generate the data necessary to estimate the concentration of a biochemical pesticide and its degradates occurring in or on various media (i.e., soil, water, air) at intervals after pesticide application. Generally these data would be submitted if adverse effects are observed in Tier I environmental effects tests or if the biochemical is applied directly to water. Figure 4 outlines the Environmental Fate testing philosophy.

Certain studies in Subdivision M refer to Subdivision N tests that specify identification of any degradation products comprising more than ten percent of the initial concentration, or in some cases levels greater than 0.01 ppm (section series 161 and 162). These studies are hydrolysis, photodegradation (soil, water, and vapor phase), aerobic soil metabolism, and aquatic metabolism studies. The Agency recognizes that application rates of many biochemical pesticides may be so low that identification of degradation products, or even calculation of percent parent material remaining, may be difficult at best. Therefore, the guidelines provide that biological monitoring techniques may be substituted for quantitative chemical analysis by instrumental methods. Specifically, this approach could be used:

- (1) To demonstrate the decline in concentration of the initially applied parent pesticide material, target organisms could be used in a bioligical monitoring test to demonstrate pesticide decline in a manner similar to efficacy testing;

- (2) To demonstrate the safety of a degradation product (mixture material and/or degradates and/or metabolites), biomonitoring tests aimed at making this determination should employ a test design similar to that required for Tier I toxicology testing (i.e., rat acute oral LD_{50}).

This is a novel approach to the problem and in many situations may be less expensive than instrumental analysis. However, standard accepted protocols for this testing approach are few, particularly regarding biomonitoring tests to demonstrate safety. Further discussion of this approach is presented in the discussion section that follows.

2. Discussion. The Tier II environmental fate testing scheme consists of twelve separate tests. Seven of these tests are identical to those described for conventional chemical pesticides in Subdivision N or Subdivision D. The remaining five tests are new. These tests, (volatility of dispensed product, dispenser-water leaching, UV (ultraviolet) absorption spectra, biomonitoring for degradation products, and biomonitoring for disappearance of biochemicals) address some of the unique properties of biochemical pesticides.

The following discussion pertains only to the five test methods unique to this subdivision. The discussion includes justification for each of the selected test methods, use of the data, and specific problems the Agency recognizes in conducting these tests.

(a) Volatility of Dispensed Product (§ 155-4). (1) Choice of Method. Volatility data are difficult to interpret due to inability to easily control such variables as temperature, surface area, and flow rate. An ideal assessment of volatility involves field volatility measurements similar to those performed by Plimmer et al., (1978) on a gypsy moth pheromone (disparlure) in a forest environment. However, such measurements are presently at the research stage and are very closely. Volatility measurements performed solely in a laboratory environment are easier and cheaper, but are not always environmentally relevant because the following factors may affect the concentration of biochemical vapor over treated areas:

Figure 4--ENVIRONMENTAL FATE TESTING PHILOSOPHY

Potential adverse effects are seen in Tier I testing. (If not, no testing is indicated in Tier II unless product has aquatic use pattern or biochemical is not applied in a controlled release device, in which case tester should proceed directly to Persistence Testing.)

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V

TRANSPORT TESTING:

Volatility (§ 155-4)
Dispenser water leaching (§ 155-5)
Vapor pressure (§ 151-17)
Water solubility (§ 151-17)

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V

Estimated Environmental Concentration (EEC) determination from mass-balance analysis.

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V

EEC indicates potential hazard. (If not, no further testing is indicated.)

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|
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V

PERSISTENCE TESTING:
Adsorption/desorption (§ 155-6)
Octanol/water partition coefficient (§ 155-7)
Hydrolysis (§ 155-9)
Aerobic soil metabolism (§ 155-10)
Aerobic aquatic metabolism (§ 155-11)
Soil photolysis (§ 155-12)
Aquatic photolysis (§ 155-13)

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V

Potential for adverse effects to nontarget organisms.
(If not, no further testing is indicated.)

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|
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V

Additional nontarget organism testing is indicated (Tier III).

- (1) Micrometeorological factors (temperature, wind speed, rainfall);
- (2) Degradation of biochemicals inside dispensers in uncontrolled environments, if applicable;
- (3) Changing rates of biochemical diffusion to dispenser surfaces, if applicable;
- (4) Vapor phase degradation; and
- (5) Adsorption to plant leaves.

The proposed method combines field and laboratory testing, allowing the assessment of factors (1), (2), and (3) above. Vapor phase degradation (4) can be measured and accounted for as described in the discussion on UV-visible absorption measurements. [See paragraph (c), below.] Unfortunately, the present state of the art on these compounds (e.g., semiochemicals) prohibits consideration of factor (5).

(2) Use of the data. Volatility data will be used in two ways. The data will be evaluated along with dispenser water leaching data, if applicable, to predict the extent of loss of biochemicals to soil or water. For example, if the volatility of a formulated biochemical is very high and the water leaching very low, it would be unlikely that a large percent of the biochemical would reach the soil. The data will also be used to estimate vapor phase concentrations near sites of application in cases where there is concern regarding beneficial insects and/or reentry hazards. Volatility is a complex issue, and the Agency invites comments on the method presented in § 155-4.

(b) Dispenser-water leaching (§ 155-5). (1) Choice of method. Standard methodology is not available which stimulates rainfall leaching biochemicals from dispensing devices. However, the method provided is one that has met with some agreement among Agency and outside experts. Although it is a novel approach, the chemistry involved is simple and classic. The method involves an eight-hour exposure of the formulated pesticide to water, followed by quantitative analysis.

Eight hours was chosen as the leaching extraction time for two reasons. First, an eight-hour exposure to water seems to be a reasonable simulation of heavy, prolonged rainfall. Second, a few hours is generally sufficient time for liquid-liquid extractions. For example, USEPA (1979) recently recommended a one-hour extraction time for the octanol/water partition coefficient. If the dispenser is constructed such that it is at least partially permeable to water, then liquid extraction will occur, since the biochemically active ingredients are usually dissolved in organic inerts.

(2) Use of the data. Dispenser-water leaching data will be used with volatility data to assess the potential for leaching of active ingredients to water or soil. If the potential for leaching is great (i.e., the estimated environmental concentration is greater than 1/5 LC_{50} or EC_{50} of the exposed non-target species), further testing may be indicated.

(c) Ultraviolet-Visible Absorption Spectra (§ 155-8). (1) Choice of methods. The methods required are very similar to those previously described by EPA (1979) in testing guidelines for new chemical substances under the Toxic Substances Control Act (44 FR 16240). The vapor phase absorption spectrum is preferred, but the liquid phase spectrum (in inert solvents) is acceptable. The methods are simple and inexpensive, and they yield useful data.

(2) Use of the data. UV-visible absorption spectra data will be used in the prediction of vapor phase half-lives when there is a potential for harm to beneficial insects. The three predominant means of atmospheric transformation are photolysis, reactions with hydroxyl radical, and reactions with ozone. Photolytic half-lives can be estimated in the following manner. The UV-visible absorbances are converted to absorption cross sections. These data are then used in combination with the solar flux and an estimate of the quantum yield to calculate the photolytic half-life. Transformation products can often be predicted, if necessary, by the scheme developed by Hendry and Kenley (1979). Rates of reaction and formation of transformation products resulting from reaction with hydroxyl radicals and ozone may also be predicted using the approach described by Hendry and Kenley (1979).

(d) Biomonitoring for disappearance of biochemicals. (1) Choice of Methods. Generally techniques very similar to those used in efficacy assessments (see section series 156) can be used to follow disappearance of biochemicals. Test methods will vary with the sites and the pests. Some relevant test methods are contained in volumes 1, 2, 3, 4, 8, and 10 of an American Institute of Biological Science (AIBS) report to EPA (1977, 1978). Pheromone activity as has been determined with the electroantennogram method (EAG) (Arn, 1975; Schneider, 1969) may not be appropriate for monitoring activities of mixtures, since only isolated peaks are examined with GC-EAG. Bioassay methods for detecting plant growth regulators in soil and water are contained in a report by Mitchell and Livingston (1968).

(2) Use of the data. The data can be used for determining half-lives of biochemicals in lieu of instrumental determinations.

(e) Biomonitoring for degradation products. (1) Choice of methods. Determination of all degradation products exceeding 10 percent of the initial pesticide concentration (as in portions of the Tier II tests that refer to tests in Subdivision N of the guidelines) can be time-consuming and costly, and may generate data that are difficult to interpret toxicologically. Thus the guidelines allow registrants to test concentrated mixtures of transformation products in the Tier I human health effects screen (§§ 152-10 through -18). If significant adverse effects are detected, further identification of transformation products may be warranted. These alternative methods apply to hydrolysis, aquatic photodegradation, and aquatic metabolism studies.

An established method for concentrating a mixture of degradation products in a toxicologically and chemically significant manner is not available. The guidelines method solicits performance of environmental fate tests at much higher concentrations than would normally be used in such studies. It is necessary to raise the concentration of these studies because maximum concentrations required in effects tests are generally higher than the maximum concentrations required in environmental fate studies.

(2) Use of the data. The data from either test chosen to be performed will be used to determine the toxicological significance of degradation products identified in environmental fate testing. The Agency welcomes comments on the relative cost effectiveness of the biomonitoring and bioassays as alternative approaches (relative to instrumental identification of reaction products) asz well as comments on their scientific merit.

(3) Tier progression. A biochemical pesticide will reach Tier II, environmental fate testing, because either.

- (1) The biochemical will be introduced directly into an aquatic environment (i.e., direct application to water), or
- (2) Adverse effects on non-target organisms are observed in Tier I.

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B. Microbial Pest Control Agents: Environmental Expression Testing.

1. Introduction. The guidelines employ the term "environmental expression" to be used with microbial pest control agents in order

to distinguish them from chemical pesticides and the term "environmental fate." Environmental expression is defined as the ability of the physiologically active component of the microbial pest control agent to propagate and become established in a new niche or host after it has been introduced (applied). The term "fate," on the other hand, has been associated with chemical pesticides and is defined in the FIFRA guidelines as the transport and transformation of a chemical by natural means after it is released to the environment from a point source, disposal site, and/or dispersive use. This definition does not apply to microbial pest control agents and, therefore, the guidelines set forth the above distinction and use of the term "environmental expression."

The tier testing scheme in section series 154 and 155 of Subdivision M is designed to present the maximum hazard to nontarget organisms in the initial (Tier I) testing of microbial pest control agents. When the Tier I maximum hazard tests yield negative results, no further testing is indicated and thus testing is kept to a minimum. Positive results (toxic or pathogenic effects) observed in Tier I will mandate further testing in Tier II, environmental expression testing.

Tier II consists of screening tests that will eliminate the need for Tier III testing whenever the results show that the agent will not become permanently established once applied or inadvertantly inserted into a new niche or host, or cannot survive except under special conditions (e.g., specific host, obligatory heterotroph).

Data development in Tier III testing requires methods that approach the state of the art in microbial ecology. Without Tier II testing, positive results in Tier I would require that the registrant proceed to the more sophisticated Tier III testing, which would probably be very costly. Thus Tier II tests can eliminate the need for intensive Tier III tests for many agents.

In Tier II, the agent is tested for ability to persist in a terrestrial, freshwater, marine, or estuarine environment so that potential exposure of nontarget organisms can be determined. For example, an agent could be saprophytic (i.e., dependent on decaying matter for nutrition in its normal environment), but could proliferate independently and unchecked in a new niche. If it can be determined that the application of the microbial pest control agent will not give it an opportunity to proliferate in a new niche, then no further testing is needed. The reason it cannot survive and grow may be the lack of an obligatory nutrient, or an absence of suitable hosts other than the target organism. If, however, there is a possibility of insertion of the microbial pest control agent into a new niche where no natural antagonists of the agent are known, then Tier III testing must be done to determine the possible toxic or pathogenic effects of such insertion.

Actual field testing (Tier IV) may be necessary to decide whether the agent can proliferate in a given environment, but because the agent is capable of permanent persistence (a property not demonstrated by chemical agents), such testing should only be undertaken after the registrant and the Agency have carefully considered the possible consequences.

The registrant may be able to identify chemical or physical markers with which the presence of the agent can be detected. Such markers could be either in the host or the environment. For example, standard laboratory procedures employ bioassay for quantitative assay of viruses, but these depend on the infective potency of the agent. However, in measuring potency of an industrial product or screening field samples, bioassay may be useless because the virus form being studied is one which is not infectious to tissue culture cell lines. There is great need for rapid and unequivocal identification of such virus forms in field samples or in any scheme designed to monitor the environment after application of a pesticidal virus. Reserach in the area may lead to development and distribution of type-specific antisera to pest management workers who can make checks on virus identity with unsophisticated equipment.

2. General information. This section outlines the scope and approach for Tier II microbial testing.

(a) Tier II testing. A microbial pesticide will reach Tier II:

- (1) Whenever Tier I testing gives positive results (toxic or pathogenic effects) in maximum hazard testing; or
- (2) Whenever the agent is a genetically engineered organism.

(b) Approach. Use pattern (terrestrial, freshwater, and/or estuarine/marine) in conjunction with positive Tier I test results will determine which one of three environmental expression tests should be conducted under Tier II testing. The tests include a greenhouse test for determining expression in a terrestrial environment and two aquaria tests for determining expression in freshwater and in estuarine/marine environments.

IX. PRODUCT PERFORMANCE DATA REQUIREMENTS

Product performance (efficacy) data requirements have been generally waived for most products. It should be noted, however, that the Administrator may require, on a case-by-case basis, efficacy data on any specific product whenever he deems that such data are necessary to make proper evaluations for decisions as to acceptability to register or to maintain or cancel registrations.

Certain efficacy-related data on biorational pest control agents have been solicited in these guidelines. These data would ordinarily be subject to the waiver policy: the available information on host spectrum and the time and the minimum effective dosage required for a product to achieve the desired level of pest control or other product performance standard. Such information would ordinarily be developed (and reported) in connection with efficacy studies and is considered important in the evaluation of nontarget organism safety data concerning biorational pesticides.

X. EXPERIMENTAL USE PERMITS

Data to support applications for experimental use permits for biorational pesticides, as described in section series 157, generally include those data that would ordinarily be developed first in preparation for product development and registration. For example, most product analysis information would be developed early in the product development stages, and the Tier I toxicology and nontarget organism toxicity tests would usually be conducted first in preparation for registration. Unless these test results indicate toxic, pathogenic, or other harmful properties, no data on residues or environmental fate would be necessary. Efficacy data follow the pattern already proposed in the Subdivision I guidelines, which waives the requirements for most products not dealing with public health areas, but § 157-4(g) also proposes the submittal of data on host spectrum, and time and minimum effective dosage required to achieve the product performance standard.

XI. PROMOTING BIORATIONAL PESTICIDES BY MODIFYING LABEL CLAIM REQUIREMENTS

Consistent with the Agency's mandate to "protect health and the environment," the EPA is currently pursuing avenues to promote the development and use of biological control agents. With publication of "Regulation of 'Biorational' Pesticides; Policy Statement and Notice of Available Background Document" (44 FR 23994, May 14, 1979), the EPA recognized "that biorational pesticides are inherently different from conventional pesticides" and that "the fundamentally different modes of action of biorationals and the consequent lower risks of adverse effects from their use" must be taken into account. Embracing this policy, with the development of Subdivision M guidelines, the Agency has endeavored to reduce the burden of extensive data development by the introduction of the tier testing concept. This departure from standard procedures is intended to function as a catalyst for development of additional innovative control agents. Those parties interested in the development of the Agency's rationale supporting the direction of its proposed policies and guidelines may refer to the above noted Federal Register Notice.

In addition to those concepts expressed within the Biorational Policy Statement, Sections 20(a) and 28 of the amended FIFRA mandate the Agency to a role supportive of biorational pesticide use. Sections 20(a) and 28 of the Act express Congress' intent for the promotion of Integrated Pest Management (IPM) and "the safe use and effectiveness of chemical, biological and alternative methods to combat and control pests...." In response to Congressional wishes, the Agency has moved forward with the funding of applicable research, the reduction of data requirements and the expeditious processing of registration applications for biorational pesticides.

The Agency recognizes, however, that thus far it has only partially tapped its arsenal of regulatory alternatives available for the promotion of those methods compatible with the desires of Congress. Public awareness of the unique qualities inherent in biorational pesticides is an integral element to the successful promotion of these agents for practical use. One of the more obvious vehicles available for reaching the public is pesticide labeling. Declaration of certain unique characteristics of these agents, those responsible for their special recognition, is currently prohibited by 40 CFR 162.10(a)(5)(ix). This section prohibits "claims as to the safety of [a] pesticide or its ingredients, including statements such as 'safe,' 'nonpoisonous,' 'noninjurious,' 'harmless' or 'nontoxic to humans and pets'...." Historical interpretation of this section has prohibited label statements concerning the lack of toxicity or effect to specific predators and parasites. The Agency could amend this section to allow claims as to lack of adverse effect on beneficial agents critical to IPM and crop production systems when supported by the appropriate data. (It is not the intent of this proposal to sanction any claims as to the safety to or lack of effect on humans, pets, or the environment, since such claims are not considered to contribute significantly to the success of programs relating to IPM. The lower degree of risk inherent in biorational pesticides will be discerned by the label signal words and the relative reduction of precautionary statements.) This amendment would be only for those agents subject to Subdivision M guidelines. Data in support of these claims would be submitted under Subdivision M guidelines, section series 154, on Non-Target Organism Hazards.

SUBDIVISION M -- GUIDELINES FOR BIORATIONAL PESTICIDES

Series 150: OVERVIEW, DEFINITIONS, AND GENERAL PROVISIONS

§ 150-1 Overview.

(a) Scope and purpose. This subdivision describes the kinds of data required by 40 CFR Part 158 to support the registration of biorational pesticide products. Biorational pesticides considered in these guidelines include both biochemical pest control agents (hereinafter called "biochemical agents") and microbial pest control agents (hereinafter called "microbial agents"). Pesticides that do not meet the definition of "biorational pesticide" (i.e., biochemical or microbial agents) as set forth in 40 CFR § 158.65 shall be tested as required by the other sections of Part 158. Certain biological control agents (e.g., macroscopic predators and parasites) are exempt from the requirements of FIFRA, as authorized by sec. 25(b) of FIFRA and specified in the Exemption from Regulation of Certain Biological Control Agents published in the Federal Register of June 2, 1982 (47 FR 23928). Generally, the testing guidelines for biochemical agents are delineated separately in this subdivision from those for microbial agents. Each section in this subdivision identifies the kinds of data required by Part 158, the standards that the studies and data should meet, and the conditions under which each kind of data is required as specified in Part 158.

(b) Exceptions to testing biorationals under Subpart M. Although certain pesticides may be biorational pesticides by definition, it may be more appropriate to test them as conventional pesticides (for biochemicals) or to require testing at higher tiers (e.g., Tier II or III) as part of the minimum testing requirements (for microbials). The following are some examples of characteristics of pesticides that may indicate that the pesticide should not be tested as an ordinary biorational pesticide:

(1) The active ingredient(s) or any of its (their) metabolites, degradation products, or impurities is (are) structurally related to a recognized carcinogen, and a theoretical worst case risk based upon the dose-response relationship for the most potent tested animal carcinogen in the chemical class exceeds the risk permitted for nitrosamines and other unintentional contaminants of pesticide products.

(2) The product is a genetically engineered microorganism with characteristics or species relationships closely allied to known pathogens that cause serious crop damage, human infections, serious diseases of domestic animals, or similar problems.

(c) Relationship to other subdivisions of the guidelines. This subdivision constitutes a complete set of guidelines for biorational pesticides. To avoid needless duplication, references are made to other subdivisions whenever the guidelines are identical. Each section series in this subdivision corresponds to a subdivision of the pesticide guidelines for conventional pesticides.

To illustrate:

Series 151 Product Analysis corresponds to Subdivision D -- Product Chemistry;

Series 152 Toxicology corresponds to Subdivision F -- Hazard Evaluation: Humans and Domestic Animals;

Series 153 Residue Analysis corresponds to Subdivision O -- Residue Chemistry;

Series 154 Nontarget Organism Hazard corresponds to a combination of Subdivision E -- Hazard Evaluation: Wildlife and Aquatic Organisms; Subdivision J -- Hazard Evaluation: Nontarget Plants; and Subdivision L -- Hazard Evaluation: Nontarget Insects;

Series 155 Environmental Fate and Expression corresponds to Subdivision N -- Environmental Fate;

Series 156 Product Performance corresponds to Subdivision G -- Product Performance;

Series 157 Experimental Use Guidelines corresponds to Subdivision I -- Experimental Use Permits; and

Series 158 Label Development corresponds to Subdivision H -- Labeling for Pesticides and Devices.

§ 150-2 Definitions.

(a) Terms used in this subdivision are defined in FIFRA, in § 162.3 of the FIFRA sec. 3 regulations, and in the following sections of the guidelines:

§ 60-2 of Subdivision D
§ 70-2 of Subdivision E

§ 80-2 of Subdivision F
§ 90-2 of Subdivision G
§ 100-2 of Subdivision H
§ 110-2 of Subdivision I
§ 120-2 of Subdivision J
§ 140-2 of Subdivision L
§ 160-2 of Subdivision N

(b) In addition, for the purposes of this subdivision:

(1) "Adjusted host equivalent" means the host equivalent times the ratio of the weight of the test organisms to the weight of the host organism.

(2) "Allomone" means a chemical emitted by one species that modifies the behavior of a different species to the benefit of the emitting species.

(3) "Aquatic animals" means all vertebrates and invertebrates that inhabit fresh, estuarine, or marine waters for all or part of their life cycles.

(4) "Aquatic use" means the use of a pesticide in a fresh water, estuarine, or marine aquatic system by either direct application or direct discharge of treated water.

(5) "Biochemical pest control agent" means a semiochemical, plant regulator, hormone, or enzyme used as a pesticide.

(6) "Biological control agent" means a living organism introduced into the environment to control the population or biological activities of another life form considered to be a pest under sec. 2(t) of FIFRA.

(7) "Biorational pesticide" means microbial pest control agents such as viruses, bacteria, protozoa, fungi and biochemical pest control agents, either naturally occurring or identical to a natural product, that attract, retard, destroy, or otherwise exert a pesticidal activity. The Agency will determine on a case-by-case basis whether synthetic biochemical agents not identical to natural biochemical agents are biorational pesticides.

(8) "Environmental expression" means the extent and manner in which a microorganism establishes and maintains its presence in an ecological niche.

(9) "Enzootic" means a disease that is present in an animal population at all times but that occurs in only small numbers of individuals at any given time.

(10) "Epizootic" means a disease attacking many animals in a population at the same time; widely diffused and rapidly spreading.

(11) "Estimated environmental concentration" means an estimate of the concentration of a biorational pesticide occurring in or on various media (i.e., soil, water, air) after pesticide application, as determined from the results of environmental fate Tier II testing (§§ 155-4 through -13) or environmental expression Tier II testing (§§ 155-18 through -20).

(12) "Genetic engineering" means to artificially alter the genetic constitution of an organism by recombinant DNA techniques.

(13) "Habitat" means the plants, animals, and physical components of the environment that constitute the natural food, physical-chemical conditions, and cover requirements of an organism.

(14) "High volatility" means that the estimated volatility of a substance based on vapor pressure and solubility in water is greater than 5×10^{-5} atm. m.3/mole ([derived from Henry's Law Constant H = vapor pressure (atm)/ water solubility (gmole pesticide/m^3 water].

(15) "Hormone" means a chemical agent, produced by a tissue or endocrine gland, that controls physiological functions or behavior of an organism.

(16) "Host equivalent" means that amount of microbial agent active ingredient that one infected host (e.g., a late instar larva) can contain.

(17) "Hydrosoil" means the sediment underlying bodies of water.

(18) "Improved strain" means an altered organism of potentially increased benefit to man created by causing a small change in the make up or sequence of the genetic material by chemical, radiation or other external mutation means.

(19) "Kairomone" means a chemical emitted by one species that modifies the behavior of a different species to the benefit of the receptor species.

(20) "Maximum expected environmental concentration" means the highest concentration of a pesticide occurring at any given time (usually immediately after application) at a site or in a medium (e.g., water, vegetation, or soil) as determined from the pesticide application rate.

(21) "Maximum hazard testing" means testing that is designed to maximize the test (non-target) organism's susceptibility to any toxic or pathogenic effects of the test substance.

(22) "Microbial pest control agent" means any of those bacteria, viruses, protozoa, and fungi that are used to control pests. Other microbial entities will be considered to be microbial pest control agents as they are developed.

(23) "Natural occurrence" means the occurrence of an organism in its normal niche where it grows, develops, and reproduces.

(24) "Naturally occurring" means the natural concentration or population of the organism or biochemical (that could also be employed as a pest control agent) present as airborne, particulate, or liquid material and that would be in a form that could result in exposure of nontarget organisms, including humans.

(25) "Niche" means the ecological position or function of an organism in a community of plants and animals.

(26) "Opportunistic pathogen" means a microorganism that takes advantage of a temporary environmental or host condition (i.e., host disability or weakness) to cause a disease in the host organism.

(27) "Passive dispensing device" means an apparatus (e.g., hollow fiber container, impregnated substrate) used to dispense a pesticide into the air through volatilization.

(28) "Pheromone" means a chemical substance produced by an organism (e.g., insects) that modifies the behavior of other individuals of the same species.

(29) "Plant regulator (natural)" means a substance produced by a plant that alters plant growth, development, and differentation.

(30) "Purest infective form" means that preparation of infective virus containing the least amount of extraneous material.

(31) "Semiochemical" means a chemical emitted by a plant or animal that modifies the behavior of receptor organisms of like or different species; semiochemicals include pheromones, allomones, and kairomones.

(32) For the purposes of section series 155 the term "substrate" means the natural material in the environment to which the microbial pest control agent is applied.

(33) "Terrestrial wildlife" means non-domestic birds or animals.

(34) "Toxin" means a poisonous substance, generated by a microorganism, plant, or animal, capable of causing toxicosis when introduced into body tissues but also capable of inducing a counteragent or antitoxin.

(35) "Typical end-use product" means a pesticide product representative of a major formulation category (e.g., emulsifiable concentrate, granular product, wettable powder) that contains the active ingredient of the registration applicant's product.

§ 150-3 General provisions.

(a) Scope. The standards contained in this section apply to all studies in this subdivision unless another section of this subdivision contains a specific standard on the same subject.

(b) Basic standards for testing. (1) Test substance for biological and environmental studies. (i) Tests requiring use of the technical grade of the active ingredient shall be conducted with the manufacturing-use product if both are identical, or with the technical grade of the active ingredient used to produce the manufacturing-use or end-use pesticide product if not identical.

(ii) The lot of the substance tested should be the same throughout the duration of the study, and the research sample should be stored under conditions that maintain purity and stability. If the stability of the test substance cannot be maintained for the duration of the study or if, for other reasons, it is not possible to use the same lot throughout the test, subsequent lots of the test substance should be selected to be as nearly identical to the original lot as practical. Chemical or biological assays should be performed to ensure composition identity and consistency.

(iii) Each lot of the test substance should be analyzed, to the limits of technical feasibility, and the name and quantities of ingredients, contaminants, and impurities listed. The determination should include the quantity of unknown material, if any, so that 100 percent of the test sample is accounted for. The test substance should be within the limits of purity, if any, certified in accordance with §§ 151-15 or -25 of this subdivision.

(iv) If the test or control substance is to be incorporated into feed or other vehicle, the period during which the test or control substance is stable in such a mixture should be determined prior to the start of the study. No mixture of test or control substance with the feed or vehicle should be maintained or used during a period exceeding the known stability of the test or control substance in the mixture. Alternatively, determinations of the stability of the test or control substance in random samples of the diet or vehicle mixture should be made at least monthly during the study to ensure that proper mixing, formulation, and storage procedures are being followed and that the appropriate concentration of the test or control substance is contained in the mixture.

(v) If the test or control substance is incorporated into feed or other vehicle, its homogeneity and concentration in the diet should be determined prior to the start of the study and each time a new mixture is prepared. Random samples of the mixture should be analyzed at least monthly to ensure that proper mixing, formulation, and storage procedures are being followed, and that the appropriate concentration of the test or control substance is contained in the mixture.

(vi) In addition to or in lieu of data otherwise specified in this subdivision, the Agency may require, after consultation with the applicant, data derived from testing to be conducted with:

(A) An analytically or microbiologically [e.g., purest infective form, (PIF) for viruses] pure grade of an active ingredient;

(B) The technical grade of an active ingredient;

(C) The labile form of infectious material (e.g., non-occluded virus);

(D) An inert ingredient of a pesticide formulation;

(E) A contaminant or impurity;

(F) A metabolite (from animals or plants) or degradation product of an active or inert ingredient;

(G) The end-use formulated product;

(H) Any additional substance (including other pesticides recommended for tank-mixing with the test substance) that enhances the toxic activity of the product for which registration is sought; or

(I) Any combination of the substances mentioned in paragraphs (b)(1)(vi)(A) through (H) of this section.

(2) Administration or application of test substance and vehicles. (i) The manner of administration or application of the test and control substance for biological or environmental testing should be selected so as to maintain accuracy of the dosage or treatment.

(ii) A vehicle used to dissolve or dilute the test substance or positive control substance should be chosen to possess the following characteristics if possible:

(A) It does not alter the absorption, distribution, metabolism, or retention of the test substance;

(B) It does not alter the chemical or biological properties of the test substance or enhance, reduce, or alter the toxic or infective characteristics of the test substance;

(C) At the levels used in the study, it does not produce physiological effects and is nontoxic; and

(D) It closely resembles the vehicle, if any, to be used under expected conditions of use of the pesticide product. It should be identical to the vehicle if possible.

(3) Controls for biological and environmental studies. Controls are used in the biological or environmental studies contained in this subdivision to ensure that observed effects are associated with the test substance exposure. The appropriate control groups should be identical in every respect to the treated groups except for exposure to the test substance. In studies involving animals or plants, all controls should (to the extent possible) be from the same source, be of the same age, receive the same care, and receive the same nutriment as the animals or plants receiving the test substance. To prevent bias, a system to randomly assign organisms (or groups) to treatment and control categories is recommended when use of such a system is possible and appropriate (double-blind study).

(i) Untreated (negative) controls. Untreated (negative) control groups are usually recommended. Untreated controls receive neither the test substance nor any ancillary material (vehicle).

(ii) Vehicle control groups. (A) If a vehicle is used to administer the test substance, a concurrent vehicle control group may be recommended. Vehicle control groups receive treatment with the vehicle alone, and the vehicle is usually administered at the highest level that the vehicle is administered in any test group in the study. Consult individual sections of this subdivision for those tests where a vehicle control is recommended.

(B) As provided in paragraph (b)(3)(ii) of this section, the vehicle should be selected on the basis of information establishing that it is non-toxic at the levels used in the study, has no independent physiological effects, and does not alter the chemistry or toxicity of the test substance. If, however, there are insufficient data on the effects of the vehicle, testing of the vehicle may be necessary.

(iii) Positive controls. Positive controls generally are not necessary. These serve as internal quality controls, and demonstrate known test organism sensitivity and response to known toxic or infective agents. They are also used to ascertain if a strain or species reacts similarly to another strain or species when exposed to the same known or standard toxicant or infective agent. Consult individual sections of this subdivision for those tests where a positive control is recommended.

(iv) Historical controls. Historical control data are when the Agency desires information on longevity, spontaneous diseases, or other characteristics of a species or strain selected for study, and for certain comparative or statistical purposes. Consult individual sections of this subdivision for those tests where historical control data are required.

§ 150-4 Reporting of data.

Each test report submitted under this subdivision should satisfy the reporting provisions of this section, unless a specific section elsewhere in this subdivision directs otherwise.

(a) General requirements. (1) Identification. Each test should identify:

(i) The name and address of the laboratory or site where the test was performed; and

(ii) Each party primarily responsible for any written or other matter contained in the report, and the portions of the report for which the party is responsible.

(2) Verification. Each test report should be:

(i) Signed by each of the senior scientific personnel, including the laboratory director responsible for performing and supervising the testing and preparing, reviewing, and approving the test report; and

(ii) Certified by the applicant or an authorized agent of the applicant as a complete and unaltered copy of the report provided by the testing laboratory, whether independent or owned, operated, or controlled by the applicant.

(b) Format and content. The test report should include all information necessary to provide a complete and accurate description and evaluation of the test procedures and results. A test report should contain at least three parts: a summary and evaluation of the test results, a description of the test procedures, and a listing of the data and information required by each applicable section of this subdivision. Metric units of measurement must be used although English units may be included. The systems may not be mixed (e.g., mg/sq. in.).

(1) Summary and evaluation of test results. This section of the test report should contain a summary and analysis of the data, and the conclusions drawn from the analysis. The summary should highlight any and all positive results or observations, and any deviations from control data values indicative of toxic effects. The summary should be presented in sufficient detail to permit independent evaluation of the results.

(2) Description of the test procedure. This section of the test report should contain a full description of the test procedure. If an applicant believes any of the reporting requirements are inapplicable, he should submit an explanatory statement to this effect. A full description of the test procedure should include but not be limited to:

(i) Deviation from standards. The report should indicate all ways in which the test procedure fails to meet applicable standards for acceptable testing contained in this subdivision, and should state the reasons for such deviations.

(ii) Test methods. Specification of test methods, including a full description of the experimental design and procedures, the length of the study, and the dates on which the study began and ended, should be stated.

(iii) Substance tested. Identification of the test substance should be provided, including:

(A) If the test substance is a chemical: chemical name, molecular structure, and a qualitative and quantitative determination of its chemical composition (including names and quantities of contaminants and impurities, within technically feasible limits. The determination should also include quantities of unknown materials, if any, to account for 100 percent of the sample;

(B) If the test substance is primarily biological: scientific name and, to the extent possible, serotype and strain or other appropriate designated type, and, to the extent possible, a qualitative and quantitative determination of composition (including names and quantities of known contaminants and impurities, within technically feasible limits). The determination should also include quantities of unknown materials, if any, to account for 100 percent of the sample;

(C) Manufacturer and lot number of the test substance, and relevant properties of the substance tested, (i.e., physical state, pH, stability, and purity); and

(D) Identification and composition of any vehicles (e.g., diluents, suspending agents, and emulsifiers) or other materials used in administering the test substance.

(iv) Animal and plant data. Animal and plant data should include:

(A) Species and strain used, reasons for selection of species (if the species is other than the species recommended by sections of this subdivision), and reasons for selection of strain;

(B) Source of supply of test organisms;

(C) Description of any pretest conditioning; (D) Method used to randomly assign animals or plants to test or control groups;

(E) Numbers of animals of each sex in each test or control group; and

(F) Age and condition of animals or plants at beginning of study.

(v) Environmental conditions. A description of the environmental conditions under which the testing was conducted should be reported. Further details may be provided by specific testing sections elsewhere in this subdivision.

(vi) Treatments or doses. For studies where test substance applications, treatments, or dosings are made, a complete description of such should be reported. Further details may be provided by specific testing sections elsewhere in this subdivision.

(vii) Treatment for diseases not caused by the test substance. In studies where test organisms have been treated with some agent or manipulated by some system to prevent or control infectious diseases not caused by the test substance a full description of such treatment or manipulation should reported. Such description should include:

(A) Identification of the test organisms affected and the disease organism involved;

(B) The nature and severity of the disease;

(C) The date of first observation and duration of the disease;

(D) The nature of the treatment or manipulation used to control or eliminate the disease, and the dates of such actions; and

(E) The outcome of the treatments in relation to the disease and to the test results.

(viii) Observations. Method, frequency, and duration of observations made during the study shall be reported. Other related specific information to be reported may be provided by specific testing sections elsewhere in this subdivision.

(ix) Availability of raw data, specimens, and samples of the test substances. The location of all raw data, specimens and samples of the test substances which are retained, and the name and address of the individual who is responsible for the archives, should be reported.

(x) References. References should be provided for the statistical and other methods employed for analyzing the data, and for any published literature used in developing the test protocol, performing the testing, making and interpreting the observations, and compiling and evaluating the results.

(3) Reporting the results and evaluation of specific tests. The test results and any evaluations of test results should be reported in accordance with the requirements of the individual specific testing sections of this subdivision. Such results and evaluations include all data, information, and analysis necessary to support the registration application and its corresponding product label claims, directions, and precautions.

(c) Statistical procedures. (1) General. Appropriate statistical methods shall be used to summarize experimental data, to express trends, and to evaluate the significance of differences in data obtained from different test groups. The methods used should reflect the current state of the art.

(2) Standard deviation and standard error. All data averages or means should be accompanied by standard deviations, to indicate the amount of variability in the data. In addition, the standard errors of the means should also be calculated, as they are useful in comparing means from different test groups; however, notations of statistically significant differences accompanied by the confidence level or probability should also be used in place of standard error determinations. Other methods of expressing data dispersion may also be used, when appropriate.

Series 151: PRODUCT ANALYSIS GUIDELINES FOR BIORATIONAL PESTICIDES

§ 151-1 General Information.

(a) Scope of product analysis requirements. (1) This section series outlines guidelines for the submittal of data and information on product analysis in support of applications for registration of both biochemical pest control agents and microbial pest control agents. These guidelines generally parallel those for conventional chemical pesticide products specified in Subdivision D.

(2) Sections 151-10 through - 18 refer to guidelines for biochemical agents and §§ 151-20 through -26 refer to guidelines for microbial pest control agents.

§ 151-2 through -9 [Reserved]

Subseries 151A: PRODUCT ANALYSIS GUIDELINES FOR BIOCHEMICAL PEST CONTROL AGENTS

§ 151-10 Product identity and disclosure of ingredients.

(a) Product identity. As required by 40 CFR § 158.165, each application for the registration of a biochemical pest control agent that is a pesticide product shall contain the product name and the trade name(s) (if different). The company code number(s) may be given.

(b) Confidential statement of formula. As required by 40 CFR § 158.165, an application for registration of a product shall contain a confidential statement of formula. The appropriate EPA form shall be used (i.e., Form 8570-4). The name of each ingredient in the product for which § 62-2 of Subdivision D specifies certified limits to be established should be listed. A separate confidential statement of formula is required for each alternate formula of a product. See FIFRA sec. 10 for requirements related to protection of trade secrets.

(c) Information on ingredients. As required by 40 CFR § 158.165, an application for registration should contain the following information (if available) on each ingredient which is listed in the confidential statement of formula required by paragraph (b) of this section:

(1) Each biochemical (including microbial toxins) should be identified by:

(i) The chemical name(s) from the Chemical Abstracts 1972-1976 Index of Nomenclature, or other well-defined name;

(ii) The Chemical Abstracts (CAS) Registry Number(s);

(iii) The structural formula(s), empirical formula(s);

(iv) The amount of biochemical present in the product in recognized units of potency or other appropriate expression of biological activity or percentage of weight;

(v) The genus and species names of the organism(s) from which the biochemical was separated or with which it is commonly associated; and

(vi) The specificity or host range of the biochemical activity and mode of action. With respect to mode of action of the biochemical, the applicant should discuss any potential hazard to man, the environment, or non-target species.

(2) Ingredients, other than biochemicals, shall be identified by:

(i) Percentage composition (by weight) of each ingredient;

(ii) Whether the ingredient is an active ingredient, an intentionally added ingredient, or an impurity;

(iii) The chemical name from the Chemical Abstracts 1972-1976 Index of Nomenclature, or other well-defined name;

(iv) The Chemical Abstracts (CAS) Registry Number;

(v) The product name, the trade name, and the common name (if established);

(vi) The experimental or internal code number;

(vii) For each active ingredient other than the biochemical, the empirical formula, and the molecular weight or the molecular weight range;

(viii) The structural formula, if it can be determined.

(3) The composition limits shall be given for each ingredient for which § 158.110 of 40 CFR Part 158 requires limits to be certified.

If space permits, this information can be listed in the confidential statement of formula; otherwise, a separate statement on certification of limits must be submitted.

§ 151-11 Manufacturing process.

As required by 40 CFR § 158.165, each product's registration must be supported by an accurate and current description of the process used to manufacture or formulate the product. The description shall contain the following information:

(a) Description of the basic manufacturing process. (1) For each biochemical derived from biological sources:

(i) The starting material shall be listed;

(ii) The steps taken, both chemical and biological, to ensure the integrity of the starting material and to limit the extraneous contamination in the unformulated biochemical shall be given;

(iii) The procedures by which the manufacturer established the identity and purity of the seed stock from which the unformulated biochemical is produced shall be described; and

(iv) The quality control methods and the techniques used to ensure a uniform or standardized product shall be reported. Unless the quality control methods are well established and recognized, they shall be submitted in detail with information regarding their accuracy, sensitivity, and interfering substances.

(2) For other ingredients, active and inert, the guidelines are those set forth in § 61-2 of Subdivision D.

(b) Toxic or sensitizing substances. If the presence of ingredients toxic or sensitizing to humans or other nontarget mammalian species is suspected at any stage of the manufacturing process, then data must be submitted to show that the substances do not exist in the final biochemical product or exist only in quantities too small to pose any hazard.

§ 151-12 Discussion on the formation of unintentional ingredients.

As required by 40 CFR § 158.165, a registration application shall include a discussion concerning potential formation and presence of unintentional ingredients in the product in quantities which may produce adverse human or environmental effects. As described under § 61-3 of Subdivision D, such unintentional ingredients may be introduced during the manufacturing process with the starting material, process solvents, equipment, packaging, and other sources; from side reactions in the manufacturing process; from interactions between ingredients; and from the degradation of ingredients. The applicant should base his discussion on established chemical theory. For biochemicals, the unintentional ingredients can include but are not limited to extraneous host residues and residues of contaminants that remain following the extraction or purification process.

§ 151-13 Analysis of samples.

A report on the results of preliminary analysis are required by 40 CFR § 158.165 to support the registration of each manufacturing-use product and those end-use products produced by an integrated formulation system. The guidelines of § 62-1 of Subdivision D regarding the analysis of samples shall apply.

§ 151-14 [Reserved]

§ 151-15 Certification of ingredient limits.

As required by 40 CFR § 158.165, each registration must be supported by a certification of ingredient limits. The guidelines of § 62-2 of Subdivision D regarding the certification of limits shall apply.

§ 151-16 Analytical methods for certified limits.

As required by 40 CFR § 158.165, information concerning analytical methods to verify certified limits are required to support the registration of each manufacturing-use product and each end-use product. The guidelines of § 62-3 of Subdivision D regarding analytical methods for certified limits shall apply.

§ 151-17 Physical and chemical properties.

As required by 40 CFR Part 158, data on physical and chemical properties are required to support the registration of each manufacturing-use product and each end-use product. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

The data pertaining to physical and chemical properties are listed in Table 1. Sections 64-1 through -21 of Subdivision D should be consulted for information on the conduct of these tests.

§ 151-18 Submittal of samples.

When required by the Agency, as provided in 40 CFR § 158.165, the applicant shall submit both a sample of the product conforming to the limits certified under § 151-15, and a sample of an analytically pure grade of the biochemical in the product. When requested by the Agency, the applicant shall also submit a sample of any additional substances in the product as listed in § 151-10(c) of this subdivision. The samples should be sent to: Chief of Chemical and Biological Investigations Branch, Benefits and Field Studies Division, OPP/OPTS (TS-768), U.S. Environmental Protection Agency, Building 402 ARC-East, Beltsville, MD 20705.

§ 151-19 [Reserved]

Table 1--SUMMARY OF PHYSICAL AND CHEMICAL DATA FOR BIOCHEMICAL PEST CONTROL AGENTS

	Test Substance		
Property	Technical or purer grade of the active ingredient	Manufacturing-use product	End-use product
Color	Yes	Yes	Yes
Physical state	Yes	Yes	Yes
Odor	Yes	Yes	Yes
Melting Point	Yes (solids)	No	No
Boiling point	Yes (liquids)	No	No
Density or specific gravity	Yes	Yes	Yes
Solubility	Yes	No	No
Vapor Pressure	Yes (pure form)	No	No
Octanol/water partition coeff.	Yes (for non-polar organics, pure form)	No	No
pH	Yes	Yes	Yes
Stability	Yes	No	No
Flammability			
-flashpoint	No	Yes	Yes
		(combustible liquids only)	
-flame extension	No	No	Yes
			(aerosols only)
Storage stability	No	Yes	Yes
Viscosity	No	Yes	Yes
		(liquids only	
Miscibility	No	Yes	Yes
		(emulsifiable liquids only)	
Corrosion characteristics	No	Yes	Yes
		(when packaged in metal, plastic, or paper containers)	

Subseries 151B: PRODUCT ANALYSIS GUIDELINES FOR MICROBIAL PEST CONTROL AGENTS

§ 151-20 Product identity and disclosure of ingredients.

(a) Product identity. As required by 40 CFR § 158.165, each application for registration of a microbial pest control agent shall contain the following information: the product name; the trade name(s) (if different). The company code number(s) may be given.

(b) Confidential statement of formula. As required by 40 CFR § 158.165, an application for registration of a product shall contain a confidential statement of formula. This statement shall include the nature and quantity of diluents and the identity and purpose of inert ingredients such as ultraviolet screens, stickers, spreaders, and other such material. The appropriate EPA form shall be used (i.e., Form 8570-4). The name of each ingredient in the product for which § 62-2 of Subdivision D requires certified limits to be established shall be listed. A separate confidential statement of formula is required for each alternate formula of a product. See Section 10 of the Act for requirements related to the protection of trade secrets.

(c) Information on ingredients. Information on ingredients is required by 40 CFR § 158.165 to support eacch application for registration. (1) The identification of bacteria, protozoa, viruses, or fungi in the product shall (to the extent possible) include the following:

(i) The taxonomic position, serotype, and strain, or any other appropriate designation. The precise test procedures and criteria used for identification [i.e., the morphological, biochemical, analytical (physical, chemical), serological, or other identification means] and the results of such tests should be provided;

(ii) The common, alternative, and superseded names;

(iii) The natural occurrence of the organism, its relationship to other species (particularly those that are pathogenic), and its history;

(iv) A description of any unusual morphological, biochemical, or resistance characteristic(s) of the organism if such characteristic(s) are different from the classic description of the organism;

(v) The amount of microbial agent present in the product in recognized units of potency, percentage of weight, units of viability or replication, or other appropriate expression of biological activity; and

(vi) The biological properties of the active agent with respect to target species, pest host range, life cycle, and mode of action. With respect to the properties of the microbial agent, any potential hazard (such as infectivity) to man, the environment, or nontarget species should be discussed.

(2) An application for registration shall contain the following information on each ingredient, other than the microbial agent, listed in the confidential statement of formula required in paragraph (b) of this section which is known to be present or which might reasonably be identified in the pesticide product.

(i) Percentage composition (by weight) of each ingredient;

(ii) Whether the ingredient is an active ingredient, an intentionally added ingredient, or an impurity;

(iii) The chemical name from the Chemical Abstracts 1972-1976 Index of Nomenclature, or other well-defined name;

(iv) The Chemical Abstracts (CAS) Registry Number;

(v) The product name, the trade name, and the common name (if established);

(vi) The experimental or internal code number;

(vii) For each active ingredient other than the microbial agents, the empirical formula, and the molecular weight or the molecular weight range;

(viii) The structural formula (when known); and

(ix) The composition limits for each ingredient for which § 62-2 of Subdivision D requires limits to be certified. If space permits, this information can be listed on the confidential statement of formula; otherwise, a separate statement on certification of limits must be submitted.

§ 151-21 Manufacturing process.

As required by 40 CFR § 158.165, each application for registration of a manufacturing-use product or end-use product should contain a description of the basic manufacturing process as required in § 61-2 of Subdivision D. The starting and intermediate materials should be listed together with the steps taken to ensure the integrity of these materials, and the steps taken to limit the extraneous contamination, both chemical and biological, in the unformulated microbial agent. This description shall include the procedures used by the manufacturer to establish the identity and purity of the culture from which the unformulated microbial agent is produced, the method of manufacture, and techniques used to ensure a uniform or standardized product. The integrity of the product as determined by the most specific and sensitive chemical or serological test must be demonstrated. If the test is not a recognized standard test, a detailed description of the test together with information regarding specificity, interfering substances, accuracy, and sensitivity must be provided.

§ 151-22 Discussion on formation of unintentional ingredients.

As required by 40 CFR § 158.165, each registration application should include the following information:

(a) Theoretical discussion. The theoretical discussion concerns the formation of each substance, aside from the control agents and intentionally added, chemically characterized active and inert ingredients, that might reasonably be present in the pesticide product, as outlined in § 61-3 of Subdivision D. Examples of such extraneous materials are: bacterial and fungal toxins, allergens, and other metabolic products; mutant strains; microbial contaminants with particular reference to potentially infective or antagonistic forms; side products from chemical reactions employed in the manufacturing process; fermentation residues from the growth of bacteria or fungi; extraneous host residues from viruses produced in cell cultures, whole animals, or other living forms; residues of contaminants that remain following the purification or extraction process; and impurities in chemicals used in the manufacturing process. The discussion shall include the procedures used to ensure the purity of the unformulated microbial agent; if purity (within reasonable limits) cannot be achieved, then the means of controlling contaminant levels to an acceptable limit must be delineated.

(b) Toxic or sensitizing substances. If substances toxic or sensitizing to humans or other non-target mammalian species are known or suspected to be present at any stage of the manufacturing process, then data must be submitted to show that the substances do not exist in the final product or exist only in quantities too small to pose any hazard.

(c) Human or animal pathogens. Human or other nontarget animal pathogens such as (but not limited to) *Shigella*, *Salmonella*, and *Vibrio* must not be present in the manufacturing-use product or the end-use product.

§ 151-23 Analysis of samples.

A report on the results of preliminary analysis are required by 40 CFR § 158.165 to support the registration of each manufacturing-use product and those products produced by an integrated formulation system.

The guidelines of § 62-1 of Subdivision D regarding the analysis of samples shall apply, with the exception that a quantitative serological or other appropriate test of the microbial agent may be substituted for the chemical analysis.

§ 151-24 [Reserved]

§ 151-25 Certification of ingredient limits.

As required by 40 CFR § 158.165, each registration must be supported by a certification of ingredient limits. The guidelines of §§ 62-2 and 62-3 of Subdivision D regarding certification of limits and analytical methods, respectively, shall apply. The limits for microbial agents should also be expressed in terms such as international units of potency per milligram when these are determined in serological or other appropriate tests.

§ 151-26 Physical and Chemical properties.

(a) When required. Data on physical and chemical properties are required to support the registration of each manufacturing-use product and each end-use product. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Substances tested. Table 2 presents the relevant data pertaining to physical and chemical properties for microbial agents. Sections 63-1 through -21 of Subdivision D should be consulted regarding the conduct and the specific provisions of the tests.

§ 151-27 Submittal of samples.

When required by the Agency, as provided in 40 CFR § 158.165, the applicant shall submit a sample of the technical grade of the active ingredient, manufacturinguse product, or end-use product. When required by the Agency, the applicant shall submit a sample of any additional substances in the product as listed in § 150-10(c) of this subdivision. The samples should be sent to: Chief of Chemical and Biological Investigations Branch, Benefits and Field Studies Division, OPP/OPTS (TS-768), U.S. Environmental Protection Agency, Building 402 ARC-East, Beltsville, MD 20705.

Table 2--SUMMARY OF PHYSICAL AND CHEMICAL DATA FOR MICROBIAL PEST CONTROL AGENTS

	Test Substance		
Property	Technical grade of the active ingredient	Manufacturing-use product	End-use product
Color	Yes	Yes	Yes
Physical state	Yes	Yes	Yes
Odor	Yes	Yes	Yes
Density or specific gravity	Yes	Yes	No
Stability	Yes	No	No
Storage stability	Yes	Yes	Yes
Viscosity	No	Yes (liquids only)	Yes
Miscibility	No	Yes (emulsifiable liquids only)	Yes
Corrosion characteristics	No	Yes (when packaged in metal, plastic or paper containers)	Yes

Series 152: TOXICOLOGY GUIDELINES FOR BIORATIONAL PESTICIDES

§ 152-1 General information.

(a) General. This section series sets forth guidelines for testing to determine the potential for detrimental effects to humans and domestic animals caused by biorational pesticides. The section series is divided into:

(1) Guidelines for the evaluation of biochemical pest control agents set forth in §§ 152-10 through -29; and

(2) Guidelines for the evaluation of microbial pest control agents set forth in §§ 152-30 through -53.

(b) Biochemical agents. Testing of biochemical agents for possible effects on humans and domestic animals is performed in a tier sequence. The potential for adverse effects can be ascertained by acute toxicity, irritation and hypersensitivity tests, by short term mutagenicity tests, and by cellular immune response studies. When detrimental effects are found in the first tier of tests, additional studies at the Tier II and III levels will be required as provided in 40 CFR § 158.165. The tier sequence and studies involved are outlined in Table 3.

(c) Microbial agents. The testing of microbial agents for possible effects on humans and domestic animals is performed in a tier sequence. These tests consist of acute toxicity/infectivity studies, cellular immune response studies, and irritation, hypersensitivity, virulence enhancement, tissue culture, teratogenicity, mutagenicity, subchronic, and chronic studies. Not all studies pertain to each organism at each tier. The general tier sequence and studies involved for microbial agents are outlined in Table 4.

§§ 152-2 through -9 [Reserved]

TABLE 3--SUMMARY OF TIER TESTS ON BIOCHEMICAL PEST CONTROL AGENTS

Test	Description of Species Tested	Test Substance[1]
TIER I		
LD50 Determination		
Oral	Rat	TG, MP, EP
Dermal	Rat, mouse, or rabbit	TG, MP, EP
Inhalation	Rat, mouse, rabbit, or guinea pig	TG, MP, EP
Irritation		
Ocular, primary	Albino rabbit	MP, EP
Dermal, primary	Guinea pig or albino rabbit	MP, EP
Hypersensitivity		
Immediate	Human experience during product development	TG, MP, EP
Non-immediate	Hamster or albino guinea pig	MP, EP
Mutagenicity Tests	Microbial organisms (see text)	TG, MP, EP
Cellular Immune Response	Mouse	TG
TIER II[2]		
Mutagenicity Tests	Mammalian cell (see text)	TG, MP, EP
Subchronic Oral	Mouse, rat or dog	TG
Subchronic Dermal	Rabbit or guinea pig (species not tested in primary Tier I test)	TG
Subchronic Inhalation	Rat	TG
Cellular Immune Response	Mouse	TG
Teratogenicity Test	Two species from rat, mouse, hamster, rabbit	TG
TIER III[2]		
Chronic Exposure	Rat	TG
Oncogenic Test	Newly weaned mouse; newly weaned rat	TG

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1. Abbreviations used: MP = manufacturing-use product; EP = end-use product; TG = technical grade or representative technical grade of active ingredient.
2. Not all tests may be indicated for each biochemical pest control agent; the appropriate tests will depend on the results of Tier I and/or Tier II tests.

TABLE 4--SUMMARY OF TIER TESTS ON MICROBIAL PEST CONTROL AGENTS

Test	Description of Species Tested and Information Concerning Test	Test Substance[1]			
		Bacteria	Fungi	Virus	Protozoa
TIER I					
LD50 Determination					
Oral	Rat	TG, EP, MP	TG, EP, MP	TG, EP, MP	TG, EP, MP
Dermal	Rat or mouse	TG, EP, MP	TG, EP, MP	TG, EP, MP	TG, EP, MP
Inhalation	Mouse, rabbit, or guinea pig	TG, EP, MP	TG, EP, MP	TG, EP, MP	TG, EP, MP
Infectivity					
Intravenous	Newly weaned mouse and hamster	TG[2]		PIF[2]	
Intracerebral	Newborn mouse; newborn hamster			PIF[2]	
Intracerebral	Mouse and rabbit				TG[2]
Intraperitoneal	Mouse and rabbit				TG[2]
Intraperitoneal	Mouse and one other species		TG[2]		
Irritation					
Dermal, primary	Guinea pig or rabbit	EP, MP	EP, MP	EP, MP	EP, MP
Ocular, primary	Rabbit	EP, MP	EP, MP	EP, MP	EP, MP
Hypersensitivity					
Immediate	Human experience during product development	TG, MP, EP	TG, MP, EP	TG, MP, EP	TG, MP, EP
Non-immediate	Hamster or guinea pig	MP, EP	MP, EP	MP, EP	MP, EP
Non-immediate	Hamster or guinea pig	MP, EP	MP, EP	MP, EP	MP, EP
Cellular Immune Response	Mice	TG	TG	TG	TG
Tissue Culture	Various cell lines (See section on viral agents)			PIF	

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[1] Abbreviations used: EP = end-use formulated product; TG = technical grade or representative technical grade of active ingredient; PIF = purest infective form; MP = manufacturing-use product.

[2] One half of the animals in the test shall be immunodepressed.

TABLE 4--CONTINUED

Test	Description of Species Tested and Information Concerning Test	Test Substance[1] Bacteria	Fungi	Virus	Protozoa
TIER II[3]					
Acute Oral	Puppies administered large doses				MP, EP
Acute Oral	Newly weaned mouse; newly weaned hamster			MP, EP	
Acute Inhalation	A different species than used in Tier I				MP, EP
Acute Inhalation	Newly weaned mouse; newly weaned hamster			MP, EP	
Acute Interperitoneal or Intracerebral	Two species other than those used in Tier I; Half the group are immunodepressed.	TG	TG		TG
Subchronic Oral	Mice, rat, or dog; 90 day test				TG
Primary Dermal	Guinea pig; use - dilution doses	EP	EP	EP	EP
Primary Ocular	Rabbit; use - dilution doses	EP	EP	EP	EP
Cellular Immune Response	Antibody formation cell mediated response	TG	TG	TG	TG
Teratogenicity Test	Two species from rat, mouse, hamster, rabbit			TG	
Mutagenicity Tests	Mammalian cell (see text)	TG	TG	TG,PIF	TG
Virulence Enhancement	Mice or hamster; serial passage	TG	TG		

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[3] Not all tests may be indicated for each microbial pest control agent; the appropriate tests will depend on the results of Tier I and/or Tier II tests.

TABLE 4--CONTINUED

Test	Description of Species Tested and Information Concerning Test	Test Substance[1] Bacteria	Fungi	Virus	Protozoa
TIER III[3]					
Chronic Oral	Rat	TG	TG	TG	TG
Oncogenicity Test	Newly weaned mouse; newly weaned rat	TG	TG	TG	TG
Mutagenicity Test	Mammals, using the expected route of exposure for humans	TG	TG	TG	TG
Teratogenicity Test	Two species from rat, mouse, hamster, or rabbit	TG	TG	TG	TG

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[3] Not all tests may be indicated for each microbial pest control agent; the appropriate tests will depend on the results of Tier I and/or Tier II tests.

Subseries 152A: TOXICOLOGY DATA GUIDELINES FOR BIOCHEMICAL AGENTS

Group A-1: Tier I Testing

§ 152-10 Acute oral toxicity study: Tier I.

(a) When required. Data from the acute oral LD50 tests are required by 40 CFR § 158.165 to support the registration of each manufacturing use product and each end-use product unless the substance to be tested under paragraph (b) of this section is a gas or highly volatile substance that cannot be administered orally.

(b) Test standards. The test standards set forth in § 150-3 of this subdivision and § 81-1(d) through (g) of Subdivision F should be met, with the following exception:

(1) Route of administration. Intubation is the preferred method of administering the oral dose.

(c) Reporting. In addition to the information required by § 150-4 of this subdivision and § 81(h) of Subdivision F, the following should be reported:

(1) Information on gross pathology of animal tissues, organs, and fluids, with emphasis on the gastro-intestinal tract as this receives the initial challenge dose;

(2) Clinical signs of illness and toxicity such as elevated temperature, unkempt appearance, altered feeding habits, weight loss, and other signs of distress or physical depression; and

(3) Any signs of recovery from these symptoms.

(d) Tier progression. (1) If acute adverse effects (e.g., the LD50 is greater than 5 g/kg) are observed, then:

(i) The subchronic oral dosing test (§ 152-20) shall be required as specified in 40 CFR § 158.165 if either of the following criteria is met:

(A) The use for which registration application is made requires a tolerance for the pesticide or an exemption from the requirement to obtain a tolerance, or requires the issuance of a food additive regulation; or

(B) The use of the pesticide product is likely to result in repeated human exposure (e.g., from repeat applications or persistence) to the product, its active ingredient(s), metabolite(s) or degradation product(s) through the oral route; and

(ii) Teratogenicity studies (§ 152-23) shall be required as specified in 40 CFR 158.165 if any of the following criteria are met:

(A) Use of the pesticide, under widespread and commonly recognized practice, may reasonably be expected to result in significant exposure to human females; or

(B) Its use requires a tolerance or an exemption from the requirement to obtain a tolerance, or its use requires issuance of a food additive regulation.

(2) If no acute adverse effects are observed (e.g., the LD50 is greater than 5 g/kg), then no further testing is recommended.

§ 152-11 Acute dermal toxicity study: Tier I.

(a) When required. Data on the single-dose dermal LD_{50} are required by 40 CFR § 158.165 to support the registration of each manufacturing-use product and each enduse formulated product, unless the substance to be tested under paragraph (b) of this section is a gas or highly volatile substance that cannot be administered dermally.

(b) Test standards. The test standards set forth in § 150-3 of this subdivision and § 82-2(d) through (g) of Subdivision F should be met with the following exceptions:

(1) Test species. A generally recognized strain of laboratory rat, mouse, or rabbit should be tested.

(2) Number of animals and selection of dose levels. (i) A trial test is recommended for the purpose of establishing a dosing regimen which shall include one dose level higher than the expected LD_{50} and at least one dose level lower than the expected LD_{50}. If data based on testing with at least 5 animals per sex with abraded skin are submitted showing that the LD_{50} is greater than 2 g/kg for the 24-hour contact period, no further testing at other dose levels is necessary. If mortality occurs, the recommendations of paragraph (b)(ii) of this section apply.

(ii) The number of animals per dose level, and the number and spacing of dose levels should be chosen such that mortality rates between 10 percent and 90 percent are produced, in order that calculation of the LD50 (abraded skin and intact skin) of males and females with a 95 percent confidence interval of 20 percent or less can be made. At least 3 dose levels of the test substance, in addition to controls, should be tested; test groups shall contain approximately equal numbers of male and female animals.

(c) Reporting. In addition to the information required by § 150-4, the following recommendations should be met:

(1) Information on the gross pathology of animal tissues, organs, and fluids;

(2) Pathological changes to the skin receiving the initial challenge dose;

(3) Clinical signs of illness or toxicity such as elevated temperature, unkempt appearance, altered feeding habits, weight loss, and other forms of distress or physical depression; and

(4) Any signs of recovery from these symptoms.

(d) Tier progression. (1) If acute adverse effects are observed, then subchronic dermal toxicity tests (§ 152-21) shall be required as specified in 40 CFR § 158.165 when pesticide use is likely to result in repeated human skin contact with the product, its active ingredients, or their breakdown products.

(2) If no acute effects are observed (e.g., during testing, greater than 2 g/kg), then no further testing is recommended.

§ 152-12 Acute inhalation toxicity study: Tier I.

(a) When required. (1) A determination of the acute inhalation toxicity is required by 40 CFR § 158.165 to support the registration of each manufacturing-use product if:

(i) The product is a gas;

(ii) The product produces a respirable vapor; or

(iii) Twenty percent or more of the aerodynamic equivalent of the product is composed of particulates not larger than 10 microns in diameter.

(2) A determination of the acute inhalation toxicity is required by 40 CFR § 158.135 to support the registration of each end-use formulated product if:

(i) The end-use formulated product (as registered or under conditions of use) is a gas, or causes a respirable vapor; or

(ii) Twenty percent or more of the aerodynamic equivalent of the end-use product (as registered or under conditions of use) is composed of particulates not larger than 10 microns in diameter.

(b) Test standards. The test standards set forth in § 150-3 of this subdivision and § 81-3(e-g) of Subdivision F should be met with the following exception:

(1) Species. Testing should be performed with laboratory strains of the rat, mouse, rabbit, or guinea pig.

(2) Equipment. The particle size created by the disseminating device must be in the respirable range for the species under test.

(c) Reporting. In addition to the information recommended by § 150-4 of this subdivision and § 81-3(h) of Subdivision F, the following should be reported:

(1) Information on the gross pathology of animal tissues, organs, and fluids;

(2) Clinical signs of illness or toxicity such as elevated temperature, unkempt appearance, altered feeding habits, weight loss, and other signs of distress and physical depression; and

(3) Any signs of recovery from these symptoms.

(d) Tier progression. (1) If acute effects are observed, then the subchronic inhalation toxicity study (§ 158.135) would be required as specified in 40 CFR § 158.165, when pesticide use may result in repeated inhalation exposure at a concentration which is likely to be toxic as determined from results of the acute inhalation testing and other testing.

(2) If no acute effects are observed (e.g., the LC50 is greater than 5 mg/liter for 4 hours duration), then no further testing is recommended.

§ 152-13 Primary eye irritation study: Tier I.

(a) When required. (1) General requirement. Data on primary eye irritation are required by 40 CFR § 158.165 to support the registration of each manufacturing-use product and each end-use product.

(2) Corrosive pesticides. Data which demonstrate that the test substance specified by paragraph (a) of this section has a pH of less than 2 or greater than 11.5 may be submitted in lieu of data from a primary eye irritation study conducted in accordance with paragraph (b) of this section. For all regulatory purposes, the Agency will assume that such a substance is corrosive.

(3) Dermal irritation data. When studies conducted with the test substance in accordance with § 152-14 indicate severe dermal irritation, the data for that section will suffice to meet the requirements of this section; the Agency will assume that a severe dermal irritant will be a severe eye irritant.

(b) Test standards. The test standards set forth in § 150-3 of this subdivision and § 81-4(e & f) of Subdivision F should be met, with the following exceptions:

(1) Test species. Testing should be performed on the albino rabbit. Selection of other mammalian species may be acceptable, but should be justified.

(c) Reporting. The reporting requirements set forth in § 150-4 of this subdivision and § 81-4(g) of Subdivision F should be met.

§ 152-14 Primary dermal irritation study: Tier I.

(a) When required. (1) General requirement. Data on primary dermal irritation are required by 40 CFR § 158.165 to support the registration of each manufacturing-use product and each end-use product.

(2) Corrosive pesticides. Data which demonstrate that the test substance specified by paragraph (a) of this section has a pH of less than 2 or greater than 11.5 may be submitted in lieu of data from a primary dermal irritation study conducted in accordance with paragraph (b) of this section. For all regulatory purposes, the Agency will assume that such a substance is corrosive.

(b) Test standards. The test standards set forth in § 150-3 of this subdivision and § 81-5(e) and (f) of Subdivision F should be met, with the following exceptions:

(1) Test species. Testing should be performed on either the albino rabbit or the guinea pig. Selection of other mammalian species may be acceptable, but should be justified.

(c) Reporting. The reporting requirements set forth in § 150-4 of this subdivision and § 81-5(g) of Subdivision F should be met.

§ 152-15 Hypersensitivity study: Tier I.

(a) When required. Data on hypersensitivity are required by 40 CFR § 158.165 to support the registration of each manufacturing-use product and of each end-use prodduct whose use will result in repeated human skin contact under conditions of use.

(b) Test standards. The test standards set forth in § 150-3 of this subdivision and § 81-6(e) and (f) of Subdivision F shall be met, with the following exceptions:

(1) Species. The test should be performed on at least one mammalian species. The albino guinea pig and hamster are the preferred species.

(2) Age and sex. Young adult males should be used when albino guinea pigs are tested. Young adults of either sex may be used when hamsters are tested.

(c) Reporting. The reporting requirements are the same as those set forth in § 150-4 of this subdivision and § 81-6(g) of Subdivision F.

§ 152-16 Hypersensitivity incidents: Tier I.

(a) When required. Data on incidents of hypersensitivity to humans or domestic animals that occur during the production or testing of the technical chemical, the manufacturing-use product, or end-use product shall be reported as required by 40 CFR § 158.165 with the toxicology data supplied in support of an application for registration. For reporting of incidents taking place after registration, refer to the requirements in sec. 6(a)(2) of FIFRA.

(b) Reporting. The reporting provisions for these incidents shall be the same as those for conventional chemical pesticides, as specified in the Pesticide Incident Report form (EPA form number 8550-5, OMB number 158-R0008). The following information shall be provided, if available:

(1) The name of the biochemical agent;

(2) The length of exposure to the agent;

(3) The time, date, and location of exposure to the agent;

(4) The situation or circumstances under which exposure to the agent occurred.

(5) Clinical observations.

§ 152-17 Studies to detect genotoxicity: Tier I.

(a) When required. (1) Data derived from short-term microbial mutagenicity tests are required by 40 CFR § 158.165 to support the registration of each end-use product that meets any of the criteria listed under (i)-(iii) below, and each manufacturing-use product which may legally be used to formulate such an end-use product. See 40 CFR 158.50 and 158.165 to determine whether these data must be submitted; Section II-B of this Subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(i) The use for which registration application is made requires a tolerance or an exemption from the requirement to obtain a tolerance, or requires issuance of a food additive regulation; or

(ii) The pesticide product is likely to result in significant human exposure by inhalation or dermal routes before or during the normal reproductive portion of the human lifespan; or

(iii) The active ingredient(s) or any of its (their) metabolites is (are) structurally related to a known mutagen or oncogen, or belong(s) to any chemical class of compounds containing known mutagens or oncogens. (Examples of chemical classes containing known mutagens are alkylating agents, N-nitroso-compounds, polynuclear aromatics, heterocyclic hydrocarbons, certain natural products such as aflatoxins, halogenated ethers and halohydrins, nucleic acid analogs, aromatic amines, azo dyes, and nitro derivatives).

(b) General test standards. The applicable test standards set forth in § 150-3 of this subdivision and § 84 of Subdivision F as well as the following test standards apply to the conduct of all studies necessary to produce the data outlined by this section:

(1) Test substance. (i) The technical grade of the active ingredient or the manufacturing-use product shall be tested to support the registration of a manufacturing-use product or an end-use product.

(2) Tests to be performed. The mutagenicity tests should include tests appropriate to address the following three categories:

(i) Gene mutations;
(ii) Structural chromosomal aberrations; and,
(iii) Other genotoxic effects as appropriate for the test substance, e.g., numerical chromosome aberrations, direct DNA damage and repair.

Currently recognized tests for each of these categories are identified in § 84 of Subdivision F. Because of the rapid improvements in this field, registrants are encouraged to discuss with the Agency: testing battery selection, protocol design and results of preliminary testing.

(3) Replication. All tests should be repeated at least once for reproducible determinations of response.

(4) Number and range of dose levels. (i) A suitable range of concentrations should be used, including at least three concentrations such that the lowest produces no effect (insignificant difference from control) and the highest induces some toxicity (if possible) to the test organisms.

(ii) For substances showing positive results, it is necessary to obtain reproducible dose-response curves in a narrow range of doses, if this is possible.

(5) Positive control groups. Concurrent positive control substances should be selected for each test, in order to assure both the sensitivity of the indicator organisms and the function of the metabolic activation system.

(6) Negative control groups. Concurrent negative controls should include the solvent, and, in addition, the test should include either a concurrent non-solvent negative control or a historical documentation for maintenance of genetic integrity of the indicator organisms.

(c) Specific test standards for the tests. Each test should be performed in accordance with the applicable standards described in § 84 of Subdivision F.

(d) Reporting. The reporting requirements set forth in § 150-4 of this subdivision should be followed.

(e) Tier progression. (1) If mutagenic effects are observed in any microbial test, then short-term mammalian mutagenicity tests (§ 152-19) shall be required as specified in 40 CFR § 158.165. Consultation with the Agency is suggested to determine which tests are to be performed, as this decision may be contingent upon which microbial tests give positive results.

(2) If mutagenic effects are not obtained, as defined by the standards of § 84 of Subdivision F, then no further testing is required by 40 CFR § 158.165.

(g) References. Refer to § 84-5 of Subdivision F.

§ 152-18 Cellular immune response studies: Tier I.

(a) When required. Data on cellular immune response as determined from tests listed in paragraph (b) are required to support the registration of each manufacturing-use product and each end-use product. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Test description. The following tests of effect(s) on immunocompetence are included in the cellular immune response studies:

(1) Blood cell counts;

(2) Leukocyte number and cell classes, including T and B cells;

(3) Functional activity of blood leukocytes;

(4) Macrophage number and function; and

(5) Serum protein determination.

(c) Test standards. In addition to the test standards set forth in § 150-3 of this subdivision, the following standards should be followed:

(1) Test substance. The technical grade of each active ingredient of the biochemical agent should be tested.

(2) Dosage. At least three dose levels of the biochemical should be tested.

(3) Species. The test should be performed using appropriate strains of the adult male and female laboratory mouse.

(4) Test methods. The following methods should be used for each test:

(i) Blood cell count. Three groups of 10 male and female mice each are separately injected (intraperitoneal) with 0.5 ml containing a test substance undiluted, diluted 10x, or diluted 100x, respectively. Three groups of 5 mice each similarly injected with physiological saline should serve as controls. Routine blood counts should be performed at 15 and 30 days after exposure. Standard hemocytometer assays should be used to ascertain the total number of peripheral blood leukocytes as well as differential counts.

(ii) Leukocyte responses and T and B cell numbers. Three groups of 10 male and female mice each are separately injected (intraperitoneally) with 0.5 ml containing a test substance undiluted, diluted 10x, or diluted 100x, respectively. Three groups of 5 mice each similarly treated with physiological saline should serve as controls. Fifteen and 30 days after treatment, treated and control mice are to be tested for absolute peripheral blood T and B lymphocyte numbers, and/or their ratio, in order to ascertain whether or not there has been a significant shift in the populations of these cells. For determining B cells, several standard assays are available such as the immunofluorescent antibody test for surface IgG containing leukocytes. These tests should be used to detect alterations in the ratio of peripheral blood leukocytes having surface markers. Alternatively, sheep red blood cell rosette assays with antibody sensitized erythrocytes or similar indirect assays for cells with receptors for immunoglobulins could be used. For the T cell, indirect immunofluorescent assays using standard techniques should also be used. For example, antisera with reactivity to specific mouse lymphocyte surface antigens (theta) will be used in a standard fluorescent antibody test.

(iii) Functional activity. Functional activity of blood leukocytes from test animals should be determined in regard to responsiveness to plant mitogens such as phytohemagglutinin and/or concanavalin A as indicators of T cell activity, and to B cell mitogens such as endotoxin (bacterial lipopolysaccharide or dextran sulfate). Give each of three groups of 10 male and female mice intraperitoneal injections of 0.5 ml undiluted test substance, test substance diluted 10X, or test substance diluted 100X, respectively. Three groups of 5 mice each similarly injected with physiological saline should serve as controls. Control and treated mice should be tested at various times thereafter, for example, at 15 and 30 days. After sacrifice, peripheral leukocytes or splenocytes, and standard numbers of washed cells (approximately 10^6 viable cells/culture) stimulated with at least two doses of the above-named mitogens in triplicate should be assessed for tritiated thymidine uptake by standard techniques. The effect of the test substance on the expected leukocyte transformation (i.e., blastogenesis) response of lymphoid cells to the mitogens should be compared for control and test substance-treated animals to determine whether there are any significant differences.

(iv) Macrophage number and function. Approximately 10 male or female mice should be injected (intraperitoneal) with 0.5 ml of undiluted test substance. The number and percent of peritoneal and/or splenic macrophages should be determined by standard phagocytic index tests. Treated animals should be tested at least twice after exposure (for example, at 15 and at 30 days) by:

(A) Determining the number and percent of peritoneal and splenic macrophage uptake of latex particles, bacteria or yeast; or

(B) Microscopic examination for percent of peritoneal and splenic macrophages that adhere to glass plates; and

(C) The number of cultured adherent cells that show esterase activity by means of histochemical stains.

(v) *Serum protein determination*. Approximately 10 male and female mice should be injected (intraperitoneal) with 0.5 ml of undiluted test biochemical. Seven and 15 days after exposure, serum protein levels should be determined by standard radial gel-diffusion assays and by electrophoresis to measure any overt effect(s) on such levels. Immunoelectrophoresis can be used to measure effects on concentrations of the different classes of immunoglobulins found in the gamma fraction of serum such as Ig G, A, M, and D.

(d) *Reporting*. The reporting requirements set forth in § 150-4 of this subdivision and § 80-4 of Subdivision F should be met.

(e) *Tier progression*. (1) If an indication of abnormality is observed in any of the tests listed in paragraph (b) of this section, then the applicable Tier II cellular immune response studies (§ 152-24) shall be required as specified in 40 CFR 158.165.

(2) If no abnormality is observed in any of the tests listed in paragraph (b), then no further testing is recommended.

(f) *References*. The following references provide useful information in developing acceptable protocols for cellular immune response studies.

(1) Bloom, B.R., and J.R. David. 1976. *In vitro* Methods in Cell-Mediated and Tumor Immunity. Academic Press, Inc., New York.

(2) Brunner, K.R., H.D. Engers, and J. Cerottini. 1976. The Cr release assay as used for the quantitative measurement of cell-mediated cytolysis *in vitro*. Pp. 423-428 *in In vitro* Methods in Cell-Mediated and Tumor Immunity. B.R. Bloom and J.R. David, eds., Academic Press, Inc., New York.

(3) Capel, P.J.A., W.P.M. Tamboer, R.M.W. DeWaal, J.L.J. Jansen, and R.A.P. Koene. 1979. Passive enhancement of mouse skin all-grafts by alloantibodies is Fc dependent. *J. Immunol*. 122:421-425.

(4) Fishbein, L. 1979. Studies in Environmental Science 4: Potential Industrial Carcinogens and Mutagens. Elseiver Scientific Publishing Co., New York.

(5) Garvey, J.S., N.E. Cremer, and D.H. Sussdorf. 1977. Methods in Immunology. W.A. Benjamin, Inc., Reading, MA.

(6) Harrington, J.R. 1974. Macrophage migration from an agarose droplet: a micro-method for assay of delayed hypersensitivity in the mouse. Cell. Immunol. 12:476-480.

(7) Jerne, N.K., and A.A. Nordin. 1963. Plaque formation in agar by single antibody-producing cells. Science 140:405.

(8) Koski, I.R., D.G. Poplack, and R.M. Blaese. 1976. A nonspecific esterase strain for the identification of monocytes and macrophages. Pp. 359-362 *in* *In vitro* Methods in Cell-Mediated and Tumor Immunity. B.R. Bloom and J.R. David, eds. Academic Press, Inc., New York.

(9) Leijh, P.C.J., M.R. Van Den Barselaar, and R. Van Furth. 1977. Kenetics of phagocytosis and intracellular killing of *Candida albicans* by human granulocytes and monocytes. Infect. Immu. 17:313-318.

(10) Ling, N.R., and J.E. Kay. 1975. Lymphocyte stimulation. Elsevier, Amsterdam.

(11) Mathe, G. 1976. Cancer active immunotherapy, Vol. 55. Recent results in cancer research. Springer-Verlag, New York.

(12) Pepys, M.B., C. Sategna-Guidetti, and D.D. Marjah. 1976. Enumeration of immunoglobulin-bearing lymphocytes in whole peripheral blood. Clin. Exp. Immuno. 26:91-94.

(13) Rose, N.R., and H. Friedman. 1976. Manual of Clinical Immunology. American Society for Microbiology, Washington, D.C.

(14) Russell, E.S., and S.E. Bernstein. 1966. Blood and blood formation. Pp. 351-372 *in* Biology of the Laboratory Mouse. E.L. Green (ed.), McGraw-Hill Book Company, New York.

(15) Schnyder, J., and M. Baggiolini. 1978. Role of phagocytosis in the activation of macrophages. J. Exp. Med. 14:1449-1457.

(16) Territo, M.C., D.W. Golde, and M.J. Cline. 1976. Macrophage activation and function. Pp. 142-147 *in* Manual of Clinical Immunology. N.R. Rose and H. Friedman, eds. American Society of Microbiology, Washington, D.C.

(17) Werner, G.H., R. Maral, F. Floc'h, and M. Jouanne. 1977. Toxicological aspects of immunopotentiation by adjuvants and immunostimulating substances. Bulletin de L'Institut Pasteur 75: 5-84.

(18) Winchester, R.J., S.M. Fu, and T. Hoffman. 1975. IgG on lymphocyte surfaces: technical problems and the significance of a third cell population. *J. Immunol*. 114:1210-1212.

Group A-2: Tier II Testing

§ 152-19 Mammalian mutagenicity tests: Tier II.

(a) *When required*: (1) Data from short-term mammalian mutagenicity tests are required by 40 CFR 158.165 to support the registration of each manufacturing-use product if positive results were obtained in any one of the Tier I microbial tests (§ 152-17) conducted to support registration of the manufacturing-use product.

(2) Data from short-term mammalian mutagenicity tests are required to support the registration of each end-use product if positive results were obtained in any one of the Tier I microbial tests (§ 152-17) conducted to support registration of the end-use product.

(3) Prior consultation with the Agency is suggested to determine which of these tests must be performed. This would be contingent upon which Tier I Microbial Test(s) gave positive results. For *example*, if the Tier I test listed below in column "I" was positive, then corresponding Tier II tests in column "II" would be required as follows:

A. Positive Tier I Test		B. Example of a Required Tier II Test
(A) Bacterial assay for	----->	Mammalian cell gene mutation
(A) Bacterial assay for reverse mutation (Ames)	----->	Mammalian cell gene mutation assay
(B) Prophage induction assay in lysogenic *E. coli*	----->	Mammalian cell transformation assay
(C) DNA damage/repair assay in *E. coli*	----->	*In vivo* mammalian cytogenetic assay
(D) Yeast mitotic recombination assay	----->	*In vitro* mammalian cell cytogenetic assay

See 40 CFR 158.50 and 158.165 to determine whether these data must be submitted; Section II-B of this Subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) General test standards. The general provisions for testing as set forth in § 150-3 of this subdivision, as well as the following test standards, apply to the conduct of all studies necessary to produce the data outlined in this section.

(1) Test substance. (i) The technical grade of the active ingredient or the manufacturing-use product shall be tested.

(2) Tests to be performed. The required mammalian mutagenicity assays may include tests appropriate to address one or more of the following three categories depending on results of the Tier I microbial assays:

(i) Gene mutations;

(ii) Structural chromosomal aberrations; and,

(iii) Other genotoxic effects as appropriate for the test substance, e.g., numerical chromosome aberrations, direct DNA damage and repair.

Currently recognized tests are specified in § 84 of Subdivision F. Because of the rapid improvements in this field, registrants are encouraged to discuss with the Agency: testing battery selection, protocol design and results of Tier I testing.

(3) For substances showing positive results, it is necessary to obtain reproducible dose-response curves in a narrow range of dosages, if possible.

(4) Replication. Each of the tests selected according to the criterion of paragraph (a)(1) above should be repeated at least once for reproducible determination of response.

(5) Route of administration. For *in vivo* assays, the route of administration should be that corresponding to potential human exposure.

(6) Positive control groups. Concurrent positive control substances should be selected for each test in order to ensure the sensitivity of the indicator organisms as well as the function of the metabolic activation system for *in vitro* assays.

(7) Negative control groups. Concurrent negative controls should include the solvent, and, in addition, the test should include either a concurrent non-solvent negative control or a historical documentation for maintenance of genetic integrity of the indicator organisms.

(c) Specific tests standards. Each test should be performed in accordance with the applicable standards described in § 84 of Subdivision F.

(d) Reporting. The applicable reporting requirements set forth in § 150-4 of this subdivision and § 84 of Subdivision F should be met.

(e) Tier progression. (1) If mutagenic effects (as defined by positive results) are found in any one of the short-term mammalian assays, then the Tier III oncogenicity studies (§ 152-29) are required as specified by 40 CFR § 158.165.

(2) If mutagenic effects are not obtained, then no further testing is recommended.

(f) References. Refer to § 84-5 of Subdivision F. In addition, the following references are provided for the mammalian cell transformation assay.

(1) General:

(i) Berwald, Y., and L. Sachs. 1963. *In vitro* cell transformation with chemical carcinogens. *Nature* 200: 1182-1184.

(ii) Butterworth, B.E. 1979. Recommendations for practical strategies for short-term testing for mutagens/carcinogens. Pp 89-102 *in* Strategies for Short-term Testing for Mutagens/Carcinogens. B. Butterworth, ed. CRC Press, West Palm Beach, Fla.

(iii) Heidelberger, C. 1975. Chemical carcinogenesis. *Ann. Rev. Biochem.* 44:79-121.

(iv) Krahn, D.F. 1979. The use of cultured mammalian cell transformation systems to identify potential carcinogens. Pp 55-66 *in* Strategies for Short-term Testing for Mutagens/Carcinogens. B. Butterworth, ed. CRC Press, West Palm Beach, Fla.

(2) HEC (Primary Syrian hamster embryo):

(i) Berwald, Y. and L. Sachs. 1965. *In vitro* transformation of normal cells to tumor cells by carcinogenic hydrocarbons. *J. Nat. Cancer Inst.* 35:641-661. (Original description of technique.)

(ii) Casto, B.C., N. Janosko, and J.A. DiPaolo. 1977. Development of a focus assay model for transformation of hamster cells *in vitro* by chemical carcinogens. *Cancer Res.* 37:3508-3515. (Detailed description of focus assay.)

(iii) DiPaolo, J.A., P. Donovan, and R. Nelson. 1969. Quantitative studies of in vitro transformation by chemical carcinogens. J. Nat. Cancer Inst. 42:867-876. (Detailed description of colony assay.)

(iv) Pienta, R., J. Poiley, and W. Lebherz. 1977. Morphological transformation of early passage golden Syrian hamster embryo cells derived from cryo-preserved primary cultures as a reliable in vitro bioassay for identifying diverse carcinogens. Int. J. Cancer 19:642-655.

(v) Pienta, R., M. Shah, W. Lebherz, and A. Andrews. 1977. Correlation of bacterial mutagenicity and hamster cell transformation with tumorigenicity induced by 2,4-toluenediamine. Cancer Lett. 3:45-52.

(vi) Poiley, J., R. Pienta, and R. Raineri. 1976. Transformation of hamster embryo cells by N-2-acetylaminofluorene in the presence of microsomal enzymes. NCI Carcinogenesis Program, Fourth Annual Collaborative Conference, Orlando, Florida, p. 85. U.S. Dept. of Health, Education and Welfare, Public Health Service, National Institutes of Health, Bethesda, Md.

(vii) Umezawa, K., T. Hirakawa, M. Tanaka, Y. Katoh, and S. Takayama. 1978. Statistical evaluation of Pienta's in vitro carcinogenesis assay. Toxicol. Lett. 2:23-27.

(3) Ad-HEC (Adenovirus-infected Syrian hamster embryo):

(i) Hatch, G., P. Balwierz, B. Casto, and J. DiPaolo. 1978. Characteristics of hamster cells transformed by the combined action of chemical and virus. Int. J. Cancer 21:121-127.

(4) RLV-RE (Rauscher virus-infected Syrian hamster embryo):

(i) Dunkel, V., J. Wolff III, R. Pienta. 1978. In vitro transformation as a presumptive test for detecting chemical carcinogens. The Cancer Bull. 29:167-174.

(ii) Freeman, A., E. Weisburger, J. Weisburger, R. Wolford, J. Maryak, and R. Huebner. Transformation of cell cultures as an indication of the carcinogenic potential of chemicals. J. Nat. Cancer Inst. 51:799-808.

(iii) Mishra, N., C. Wilson, K. Pant, and F. Thomas. 1978. Simultaneous determination of cellular mutagenesis and transformation of chemical carcinogens in Fischer rat embryo cells. J. Toxicol. and Environ. Health 4:79-91.

(5) BHK-21 (Baby hamster kidney):

(i) Ishii, Y., J.A. Elliot, N.K. Mishra, and M. Leiberman. 1977. Quantitative studies of transformation by chemical carcinogens and ultraviolet light using a subclone of BHK_{21} Clone 13 Syrian hamster cells. Cancer Res. 37:2023-2029.

(ii) Purchase, I.F.H., E. Longstaff, E. Ashby, J.A. Styles, D.Anderson, P.A. Lefevre, and F.R. Westwood. 1978. An evaluation of six shortterm tests for detecting organic chemical carcinogens. Appendix III. Mammalian cell transformation. Brit. J. Cancer 37:931-935.

(iii) Styles, J.A. 1977. A method for detecting carcinogenic organic chemicals using mammalian cells in culture. Br. J. Cancer 36:558-563.

(6) C3H-3T3 (CH3 mouse embryo fibroblast):

(i) Kakunaga, T. 1973. A quantitative system for assay of malignant transformation by chemical carcinogens using a clone derived from BALB/3T3. Int. J. Cancer 12:463-473.

(7) Balb-10T1/2 (Balb mouse embryo fibroblast):

(i) Reznikoff, C.A., D.W. Brankow, and C. Heidelberger. 1973. Establishment and characterization of a cloned line of C3H mouse embryo cells sensitive to postconfluence inhibition of division, Cancer Res. 33: 3231-3238. (Origin of C3H 10T1/2.)

(ii) Reznikoff, C.A., J.S. Berstram, D.W. Brankow, and C. Heidelberger. 1973. Quantitative and qualitative studies of chemical transformation of cloned C3H mouse embryo cells sensitive to postconfluence inhibition of cell division. Cancer Res. 33:3239-3249.

§ 152-20 Subchronic oral dosing studies: Tier II.

(a) When required. Data from subchronic oral dosing studies are required by 40 CFR § 158.165 to support the registration of each end-use product for which acute adverse effects were observed during acute oral toxicity studies (§ 152-10) and each manufacturing-use product which may legally be used to formulate such an end-use product when either of the criteria presented in (1) and (2) below, are met. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(1) The use for which registration application is made requires a tolerance for the pesticide or an exemption from the requirement to obtain a tolerance, or requires issuing a food additive regulation; or

(2) The use of the pesticide product is likely to result in repeated human exposure to the product, its active ingredient(s), or degradation product(s) through the oral route.

(b) Test standards. The test standards are set forth in § 150-3 of this subdivision and § 82-1(c) of Subdivision F.

(c) Reporting. The reporting provisions are the same as those required in § 82-1(h) of Subdivision F.

(d) Tier progression. (1) Data on a chronic exposure study (§ 152-26) are required by 40 CFR 158.165 if the potential for adverse chronic effects are indicated based on:

(i) The subchronic effect level established in this study;

(ii) The pesticide use pattern (e.g., rate, frequency, and location of application); and

(iii) The frequency and level of repeated human exposure that is expected.

(2) Data on an oncogenicity study (§ 152-29) are required by 40 CFR 158.165 if the test results of this study reveal a morphologic effect (e.g., hyperplasia, metaplasia) in any organ that potentially could lead to neoplastic change.

(3) If the potential for chronic adverse effects is not indicated by paragraph (d)(1)(i),(ii), and (iii) of this section, and no morphological effects are noted (in any organ) that potentially could lead to neoplastic change, then no additional testing is recommended.

§ 152-21 Subchronic dermal toxicity study: Tier II

(a) When required. Data from the subchronic dermal toxicity studies are required by 40 CFR § 158.165 to support the registration of each end-use product for which acute adverse effects were observed during acute dermal toxicity studies (§ 152-11) and each manufacturing-use product which may legally be used to formulate such an end-use product and when the pesticide use is likely to result repeated human skin contact with the product, its active ingredients, or its breakdown products. See 40 CFR § 158.50 and § 158.165 to determine whether thes[illegible]ta must be submitted;

Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Test standards. The test standards are set forth in § 150-3 of this subdivision and § 82-2(b) of Subdivision F.

(c) Reporting. The reporting provisions are the same as those for testing conventional chemical pesticides as set forth in § 82-2(h) of Subdivision F.

(d) Tier progression. (1) Data on a chronic exposure study (§ 152-26) are required by 40 CFR 158.165 if a potential for adverse chronic effects is indicated, based on:

(i) The subchronic effect levels established in this study;

(ii) The pesticide use pattern (e.g., rate, frequency, and site of application); and

(iii) The site, frequency, and level of repeated human exposure that is expected.

(2) Data on an oncogenicity study (§ 152-29) are required by 40 CFR § 158.165 if the test results of this study reveal a morphologic effect (e.g., hyperplasia, metaplasia) in any organ that potentially could lead to neoplastic change.

(3) If the potential for chronic adverse effects is not indicated based on paragraph (d)(1)(i), (ii), and (iii), and no morphologic effects are noted (in any organ) that potentially could lead to neoplastic change, then no further testing is recommended.

§ 152-22 Subchronic inhalation toxicity study: Tier II.

(a) When required. Data from the subchronic inhalation studies are required by 40 CFR § 158.165 to support the registration of each end-use product for which acute adverse effects were observed during the acute inhalation study (§ 152-12) and each manufacturing-use product which may legally be used to formulate such an end-use product and when pesticidal use may result in repeated inhalation exposure at a concentration which is likely to be toxic as determined from results of the acute inhalation testing and other testing. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this Subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Test standards. The test standards set forth in § 150-3 of this subdivision and § 82-4(c) of Subdivision F should be met, with the following exceptions:

(1) Equipment. The particle size created by the disseminating device must be in the respirable range for the species under test.

(2) (Reserved)

(c) Reporting. The reporting provisions are the same as those set forth in § 82-4(g) of Subdivision F.

(d) Tier progression. (1) Data on a chronic exposure study (§ 152-26) are required by 40 CFR § 158.165 if the potential for adverse chronic effects is indicated, based on:

(i) The subchronic effect levels established in this study;

(ii) The pesticide use pattern (e.g., rate, frequency, and site of application), and

(iii) The site, frequency, and level of repeated human exposure that is expected.

(2) Data on an oncogenicity study (§ 152-29) are required by 40 CFR § 158.165 if the test results of this study reveal a morphologic effect (e.g., hyperplasia, metaplasia) in any organ that potentially could lead to neoplastic change.

(3) If the potential for chronic adverse effects is not indicated based on paragraph (d)(1)(i), (ii), and (iii) of this section and no morphologic effects are noted (in any organ) that potentially could lead to neoplastic change, then no further testing is recommended.

§ 152-23 Teratogenicity studies: Tier II.

(a) When required. Data from teratogenicity studies are required by 40 CFR § 158.165 to support the registration of each end-use product for which adverse effects were observed during acute oral studies (§ 152-10) and each manufacturing-use product which may legally be used to formulate such an end-use product when either of the criteria in (1) or (2) below, are met. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(1) Use of the product under widespread and recognized practice may reasonably be expected to result in significant exposure to female humans; or

(2) Its use requires a tolerance or an exemption from the requirement for a tolerance, or its use requires issuance of a food additive regulation.

(b) Test standards. The test standards are the same as those set forth in § 83-3(b) of Subdivision F.

(c) Reporting. The reporting requirements are the same as those set forth in § 83-3(h) of Subdivision F.

§ 152-24 Cellular immune response studies: Tier II.

(a) When required. Data on cellular immune response studies (Tier II) are required by 40 CFR § 158.165 to support the registration of each manufacturing-use product and each end-use product when adverse effects are observed in the Tier I cellular immune response studies described in § 152-18 of this subdivision.

(b) Test standards. In addition to the test standards set forth in § 150-3 and § 152-18(c) of this subdivision, the following standards should be met:

(1) Test substance. The technical grade of the active ingredient(s) shall be tested.

(2) Species. Appropriate strains of the laboratory mouse should be used to perform these studies.

(3) Test methods. (i) Antibody-forming activity. Immunized mice are injected (intravenously) with the test substance and the antibody response is subsequently assayed. Groups of at least five mice each of a standard inbred strain are injected (intravenously) with the maximum practical dose of the test substance. The animals are then immunized at two time intervals after treatment (for example 15 or 30 days) with a standardized dose of an antigen such as sheep erythrocytes (for example, 4×10^6 washed red blood cells). Four days later, the spleen is removed and the number of splenic antibody plaque-forming cells (PFC) in the spleen of animals exposed to various levels of pesticides are to be determined. For this purpose the standard hemolytic antibody plaque assay for B lymphocyte responses to erythrocytes can be performed. It is not sufficient to merely determine the serum antibody levels, since such tests are relatively insensitive for detection of all but gross changes in antibody responsiveness.

(ii) Cell-mediated immune responses. To determine whether cellmediated immune (CMI) responses have been affected by the test substance, at least one assay for CMI should be performed either with antigens derived from whole animals, from mammalian cells in culture, or from other antigens. The tests should be performed for animals treated with the maximum dose of a test substance. A test should be performed with groups of at least five animals each at two time intervals after exposure, such as 15 or 30 days. Selection of the specific assays are the option of the registration applicant. A typical *in vivo* assay could be used to determine allogenic skin graft rejection time and/or resistance of treated animals to highly allogenic tumor cells. For example, groups of five mice each could be given a full thickness allogenic skin graft, and mean survival time determined in comparison to the survival time of allogenic skin grafts on untreated animals. Alternatively, animals could be injected with test tumor cells (e.g., mastacytoma or other well-studied tumor cells) in which an LD50 can be readily established for control animals. The effect of the pesticide on the resistance or susceptibility of the animals to challenge by the tumor cells in terms of altering either the LD50 or the time of rejection of the tumors could be assayed in comparison to controls. *In vitro* assays could also be performed; for example, spleen cells from animals sensitized with a normal allogenic skin graft or given a tumor cell injection could be assayed for quantitative cell-mediated immune responses (CMI) followed by a standard chromium release assay. For example, in a chromium release assay chromium-labeled target cells (of an appropriate donor strain) are exposed to splenocytes from sensitized animals *in vitro* to ascertain responsiveness of "killer" T lymphocytes present in treated animals. For such tests, 10^6 lymphoid cells are obtained from sensitized animals, either control animals, or animals treated with a maximum dose of test substance at an earlier time (e.g., day 15). In addition, lymphoid cells from treated animals could be tested for their ability to generate the "migration inhibitory factor" *in vitro*. For these tests, 10^6 splenocytes are placed in microcapillary tubes with or without a specific antigen such as the purified protein fractions of mycobacteria. Treated animals are first sensitized with the Bacillus CamilleGuerin (BCG) or mycobacteria extract. The ability of the mononuclear spleen cell suspension to migrate from the chamber in the presence of antigen can be determined and used as a correlate of cellular immunity. Many modifications of such CMI responses *in vitro* are available, including several well standardized migration inhibition type tests. In addition, a test for blastogenic responsiveness of leukocytes similar to the antigen tests could be performed. For example, in a typical test system spleen cells from treated animals are cultured in microwell chambers with and without a mitotic stimulator such as phytohemaglutinin, concanavalin A, lipopolysaccharide, or even an extract of allogenic cells. The ability of the spleen cells to respond to these mitogens *in vitro* as measured by uptake of tritiated thymidine is considered one assessment of cellular immunity.

(c) Reporting. The reporting requirements are the same as those set forth in § 150-4 of this subdivision and § 80-4 of Subdivision F.

(d) Tier progression. If adverse cellular effects suggesting oncogenic potential are observed in this study, data from an oncogenicity study (§ 152-29) are required by 40 CFR § 158.165.

(e) References. Refer to § 152-18(f).

§ 152-25 [Reserved]

Group A-3: Tier III Testing

§ 152-26 Chronic exposure study: Tier III.

(a) When required. Data on a chronic exposure study is required by 40 CFR § 158.165 to support the registration of each end-use product and each manufacturing-use product which may legally be used to formulate such an end-use product if the potential for adverse chronic effects are indicated based on:

(1) The subchronic effect levels established in the subchronic oral toxicity studies (§ 152-20), the subchronic dermal toxicity studies (§ 152-21), or the subchronic inhalation toxicity studies (§ 152-22);

(2) The pesticide use pattern (e.g., rate, frequency, and site of application); and

(3) The frequency and level of repeated human exposure that is expected.

(4) See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Combined testing. A chronic feeding study may be combined with an oncogenicity evaluation, pursuant to § 152-29, provided that standards for both types of testing are met.

(c) Test standards. The test standards set forth in § 83-1(d) and (e) of Subdivision F should be met with the exception of the following:

(1) Route of administration. The route of administration should be as similar as possible to the principal expected human exposure route.

(i) Pesticides that need a tolerance or an exemption from the requirement to obtain a tolerance or whose use requires a food additive regulation should be administered in the diet (unless some characteristic of the pesticide precludes dietary administration to test animals).

(ii) In all cases, use of routes of administration not corresponding to the principal expected human exposure routes should be justified in the test report. Factors such as absorption metabolism, and distribution of the compound following administration as well as results of previous tests should be taken into consideration in selecting a route of administration other than that corresponding to the principal expected human exposure route.

(iii) Specific test protocols for the inhalation or dermal exposure route should be discussed with the Agency prior to initiation of the test.

(d) Data reporting and evaluation. The reporting and evaluation requirements are the same as those set forth in § 83-1(f) of Subdivision F.

§§ 152-27 through -28 [Reserved].

§ 152-29 Oncogenicity studies: Tier III.

(a) When required. Data from oncogenicity testing are required by 40 CFR § 158.165 to support the registration of each end-use product and each manufacturing-use product which may legally be used to formulate such an end-use product that meets either of the following criteria:

(1) The active ingredient(s) or any of its (their) metabolites, degradation products, or impurities produce(s) in subchronic studies (§§ 152-20, -21, or -22) a morphologic effect (e.g., hyperplasia, metaplasia) in any organ that potentially could lead to neoplastic change; or

(2) If adverse cellular effects suggesting oncogenic potential are observed in cellular immune response studies (§ 152-24) or in mammalian mutagenicity assays (§ 152-19).

(b) Combined testing. An oncogenic evaluation may be combined with a chronic feeding study, pursuant to § 152-26, provided that standards for both types of testing are met.

(c) Test standards. The test standards are the same as those set forth in § 83-2(d) and (e) of Subdivision F.

(d) Reporting. The reporting provisions are the same as those set forth in § 83-2(f) of Subdivision F.

Subseries 152B: TOXICOLOGY DATA GUIDELINES FOR MICROBIAL PEST CONTROL AGENTS

Group B-1: Tier I Testing.

§ 152-30 Acute oral toxicity/infectivity study with microbial pest control agents: Tier I.

(a) When required. Acute oral toxicity/infectivity data are required by 40 CFR § 158.165 to support the registration of each manufacturing-use product and each end-use product.

(b) Test standards. In addition to the standards set forth in § 150-3 of this subdivision and § 80-3 and § 81(d) through (g) of Subdivision F, the following standards should be met:

(1) Species. Testing should be performed on the laboratory rat.

(2) Number of animals and selection of dose levels.

(i) Trial testing is recommended to establish a dose level greater than the LD50. If submitted test data using at least five animals per sex show that the oral LD50 is greater than 5 g/kg, no further testing at other dose levels is necessary. If mortality occurs, the provisions of paragraph (b)(2)(ii) of this section apply.

(ii) At least 3 dose levels spaced appropriately should be tested using adequate numbers of animals to form test groups with mortality rates in the 10 to 90 percent range in order to permit LD50 determinations for males and females with a 95 percent confidence interval of 20 percent or less.

(iii) An adequate numbers of animals per dose level in addition to those described in (b)(2)(ii) of this section should be exposed to the microbial agent so that 2 female rats and 2 male rats can be sacrificed at 1 week post-treatment to examine tissues and organs for gross pathology and presence of the viable microbial agent.

(iv) All animals should be dosed by gavage.

(3) Control animals. A concurrent group of animals treated with the vehicle containing killed organisms (autoclaved) should be included as a control in each acute oral LD50 study.

(4) Dose quantification. Titers (of the microbial suspensions administered to test animals) should be performed by plating dilutions on laboratory surface media or other suitable media or on host organisms to enumerate viable organisms.

(5) Duration of tests. Surviving exposed animals and controls should be observed for 14 days or until all signs of reversible infectivity or toxicity subsides, whichever occurs later.

(6) Conduct of test.

(i) Fasting. Food should be withheld from the animals during the night prior to dosing.

(ii) Observation. The animals should be observed frequently during the day of dosing and checked at least once each morning and late afternoon thereafter. The following should be recorded even if the animals recover completely from the exposure:

> Nature and onset of all gross or visible clinical signs of illness such as elevated temperature, unkempt appearance, altered feeding habits, weight loss, various forms of distress, and physical depression.

(iii) Examination of excreta. Urine and feces samples from the test animals should be collected at 24, 48, and 72 hours following test initiation and examined for the presence of the microbial agent.

(iv) Assay for specific antibody production. If test duration exceeds 14 days, than an assay for specific antibody production should be performed.

(v) Sacrifice and necropsy. All test animals surviving at the end of the observation period should be sacrificed. All test animals, whether dying during the test or sacrificed, should be subjected to a complete gross necropsy. In addition microorganism dissemination, replication, and survival in animal tissues, organs, and fluids should be determined, including survival in the intestinal tract. Samples should be cultured on laboratory surface media or other suitable media or host organisms to provide qualitative and quantitative measurements of survival and multiplication of the microorganism.

(c) Data reporting and evaluation. In addition to the requirements in § 80-4 of Subdivision F, the test report should include the following information:

(1) Tabulation of response data by sex and dose level (i.e., number of animals dying per number of animals showing signs of infectivity per number of animals exposed), and

(i) Time of death after dosing;

(ii) The LD50, for each sex and test substance, calculated at the end of the observation period (with method of calculation specified) expressed in numbers of viable microorganisms per kg body weight and mg test substance per kg body weight; and

(iii) Dose-response curve and slope.

(2) In addition, gross pathology, microorganism dissemination, replication, and survival in animal tissues, organs, and fluids should be reported, including survival in the intestinal tract. Results of the assay for specific antibody production should be reported, when applicable.

(3) The test organism should be characterized according to genus, species serotype and strain (according to current acceptable taxonomy), and the percentage of unknown fermentation solids or other materials present indicated to account for 100 percent of the sample.

(d) Tier progression.

(1) If evidence of infectivity, persistence, presence of viable microbial agent in test animal excreta, replication, or toxic effects are observed in the acute oral studies, then the following Tier II testing shall be required as specified in 40 CFR § 158.165:

(i) Bacteria or fungi. Acute intraperitoneal or intracerebral tests shall be conducted in two animal species other than those used in Tier I. Half of the test animals should be immunodepressed (§ 152-43). The bacterial and fungal virulence enhancement study (§ 152-48) shall be conducted.

(ii) Viruses. An acute oral infectivity study shall be conducted on newly weaned mice and newly weaned hamsters (§ 152-40), and the teratogenicity study (§ 152-47) shall be required.

(iii) Protozoa.

(A) An acute oral infectivity study in puppies should be conducted using large doses of protozoa (§ 152-40).

(B) A subchronic 90-day oral test in the mouse, rat, or dog - should be performed (§ 152-42).

(2) If evidence of acute oral infectivity, organism persistence, replication, or toxic properties (e.g., LD50 greater than 5g/mg) is not observed, additional testing will not be required.

(e) References.

(1) The following references contain information useful for developing an acceptable protocol.

(i) Fisher, R., and L. Rosner. 1959. Toxicology of the microbial insecticide thuricide. J. Agric. Food Chem. 7:686-688.

(ii) Ford, S., and L. Friedman. 1967. Experimental study of the pathogenicity of aspergilli for mice. J. Bacteriol. 94:928-933.

(iii) Forsberg, C.W. (ed.). 1976. Bacillus thuringiensis: its effects on environmental quality. Publication no. 15385 of the Environmental Secretariat, National Research Council of Canada.

(2) The following references provide information on acceptable methods of calculating the LD50:

(i) Finney, D.J. 1971. Probit Analysis. 3rd Edition. Chapters 3 and 4. Cambridge University Press. Cambridge, Eng.

(ii) Litchfield, J.T., Jr., and F. Wilcoxon. 1949. A simplified method of evaluating dose-effect experiments. J. Pharmacol. Exp. Therap. 96:99-115.

(iii) Thompson, W.R. 1974. Use of moving averages and interpolation to estimate median effective dose. Bacteriological Rev. 11:115-145.

(iv) Weil, C.S. 1952. Tables for convenient calculation of median effective dose and instruction on their use. Biometrics 8:249-263.

§ 152-31 Acute dermal toxicity/infectivity study with microbial pest control agents: Tier I.

(a) When required. Data on acute dermal infectivity are required by 40 CFR § 158.165 to support the registration of each manufacturing-use and each end-use product.

(b) Test standards. In addition to the applicable standards set forth in § 150-3 of this subdivision and § 80-3 and § 81-2(d) through (g) of Subdivision F, an acute dermal infectivity study should meet the following standards:

(1) Species. Testing should be performed with at least one mammalian species, preferably the rat or mouse.

(2) Sex and age. Young adult male and female animals should be used.

(3) Number of animals and selection of dose levels.

(i) A trial test is recommended for the purpose of establishing a dosing regimen which should include one dose level higher than the expected LD50. If data from abraded skin tests on at least 5 animals of each sex are submitted showing that the dermal LD50 is greater than 2 g/kg for the 24-hour contact period, no further testing at other dose levels is necessary. If mortality is produced, the provisions of paragraph (b)(3)(ii) of this section apply.

(ii) At least 3 dose levels spaced appropriately should be tested using adequate numbers of animals to form test groups with mortality rates in the 10 to 90 percent range in order to permit LD50 determinations (abraded skin and intact skin) for males and for females with a 95 percent confidence interval of 20 percent or less. In addition, the requirements of paragraph (b)(3) (iii) of this section may apply.

(iii) Data from tests performed with the use dilutions of a product may be necessary if the use dilution is intended for application as a mist or spray.

(4) Control animals. A concurrent untreated control group of animals should be included in the test. A concurrent vehicle control group is recommended if a vehicle or diluent used in administering the test substance is expected to elicit an important toxicologic response, or if insufficient data exists on the acute effects of the vehicle.

(5) Dose quantification. Titers of microbial suspensions to test animals should be performed by plating dilutions on laboratory surface or other suitable media or host organisms for enumeration of viable organisms.

(6) Conduct of test.

(i) Application. In all animals, the application site should be as free of hair as possible. In addition, the application sites in abraded-skin groups should be abraded in such a way as to penetrate the stratum corneum but not the dermis. The test substance must be kept in contact with skin covering at least 10 percent of the body surface for at least 24 hours. [See Draize (1944) for equivalent sq. cm. of body surface.] The preferred application site is a band around the trunk of the test animal. A wrapping material such as gauze covered by an impervious nonreactive rubberized or plastic material should be used to retard evaporation and to keep the test substance in contact with the skin. At the end of the exposure period, the wrapping should be removed and the skin wiped (but not washed) to remove remaining test substance.

(ii) Duration of observation. Animals should be observed for at least 14 days after dosing or until all signs of reversible infectivity or toxicity in survivors subside, whichever occurs later.

(iii) Observations. The animals should be observed frequently during the day of dosing and checked at least once each morning and late afternoon thereafter. The following should be recorded even though the animals recover completely from the exposure: nature and onset of all gross or visible clinical signs of illness such as elevated temperature, unkempt appearance, altered feeding habits, weight loss, various forms of physical distress, depression, and similar responses.

(iv) Assay for specific antibody production. If test duration exceeds 14 days, then an assay for antibody production should be performed.

(v) Sacrifice and necropsy. All test and control animals surviving at the end of the observation period (14 days) are sacrificed. All test animals, whether dying during the test or sacrificed, are subjected to a complete gross necropsy. In addition to gross pathology, microorganism dissemination, replication, survival in animal tissue, organs, and fluids should be determined, including survival in the skin. Samples should be cultured on laboratory surface or other suitable media or host organisms to provide qualitative and quantitative measurements of survival and multiplication of the microorganism.

(vi) Histopathology. Examination of skin should include histological examination of treated tissues in accordance with § 80-3(b)(11) of Subdivision F.

(c) Data reporting and evaluation. In addition to the applicable general information required by § 80-4 of Subdivision F, the test report should include the following information:

(1) Tabulation of response data by sex and dose level (number of animals dying per number of animals showing signs of infectivity per number of animals exposed);

(2) Time of death after dosing;

(3) Observations of signs and symptoms;

(4) Gross pathological findings;

(5) Evidence of microorganism dissemination, replication, and survival in animal tissues, organs, and fluids, including survival in skin;

(6) LD50 determinations for each sex and for each test substance for animals with abraded skin and for animals with intact skin calculated at the end of the observation period (with method of calculation specified) expressed in numbers of viable microorganisms per kg body weight and mg of test substance per kg body weight;

(7) 95 percent confidence interval for the LD_{50};

(8) Dose-response curve and slope; and

(9) Identification of the test microorganism, including:

(i) Genus, species, serotype, and strain (to the extent possible), according to current acceptable taxonomy; and

(ii) The percent of unknown fermentation solids or other materials present to account for 100 percent of the sample.

(10) Results of assays for specific antibody production, when applicable.

(d) Tier progression.

(1) No further testing is required by 40 CFR 158.165 for viruses or protozoa.

(2) If evidence of infectivity, organism persistence or replication, or toxic effects is observed following acute dermal studies with bacteria or fungi, then acute intraperitoneal or intracerebral tests shall be conducted in two animal species other than those used in Tier I (§ 152-43) as specified by 40 CFR 158.165. Half of the test animals should be immunodepressed.

(e) References.

(1) Draize, J.H., G. Woodward, and H.O. Calvery. 1944. Methods for study of irritation and toxicity of substances applied topically to skin and mucous membranes. J. Pharmacol. Exp. Ther. 83:377-390.

(2) Draize, J.H. 1965. Appraisal of the safety of chemicals in foods, drugs and cosmetics - Dermal toxicity. Assoc. of Food and Drug Officials of the United States. Topeka, Kansas. Pp. 46-59.

§ 152-32 Acute inhalation toxicity/infectivity study with microbial pest control agents: Tier I.

(a) When required. Data on acute inhalation toxicity/infectivity are required by 40 CFR § 158.165 to support the registration of each manufacturing-use and each end-use product if 20 percent or more of the aerodynamic equivalent of the product (as registered or under conditions of use) is composed of particulates under 10 microns in diameter.

(b) Test standards. In addition to the applicable standards set forth in § 150-3 of this subdivision and § 80-3 and § 81-3(e) through (g) Subdivision F, an acute inhalation LC_{50} study should meet the following standards:

(1) Species. Testing should be performed with the laboratory mouse, rabbit, or guinea pig.

(2) Sex and age. Young adult male and female animals should be used.

(3) Number of animals and selection of dose levels.

(i) A trial test is recommended for the purpose of establishing a dose regimen which should include one dose level higher than the expected LC_{50} and at least one dose level below the expected LC_{50}. If data based on testing with at least 5 animals per sex are submitted showing that the LC_{50} is greater than 5 mg. equivalent of viable microbial agent for 4 hours duration, no further testing at other dose levels is necessary. If death occurs, the requirements of paragraph (b)(3)(ii) of this section apply.

(ii) At least three dose levels should be chosen using an adequate number of animals to form test groups with mortality rates between 10 percent and 90 percent, and to permit LC50 calculations with a 95 percent confidence limit of 20 percent or less.

(4) Duration of test. In selecting the exposure period, allowance must be made for chamber concentration equilibration time. If no problems are encountered in maintaining a steady concentration of the test substance in the chamber(s), the exposure period should be at least 1 hour. If problems are encountered in maintaining a steady concentration, the exposure period should last up to 4 hours. The animals should be observed for 14 days, or until signs of reversible infectivity subside, whichever occurs later.

(5) Dose quantification. Titers of microbial suspensions administered to test animals should be calculated by plating dilutions on laboratory surface media or other suitable media or in host organisms for enumeration of viable organisms.

(6) Control groups.

(i) A concurrent untreated control group is necessary.

(ii) If any solvent, other than water, is used in generating the exposure atmosphere, a vehicle control group is necessary. The vehicle control group should be exposed to an atmosphere containing the greatest concentration of solvent present in any test system.

(7) Exposure chamber design and operation.

(i) Inhalation exposure techniques described in this section are based on the use of whole-body inhalation chambers that allow experimental animals to receive whole-body dermal exposure and possibly large oral exposure, as well as exposure by inhalation. In some cases, the investigators will wawnt to use other inhalation exposure techniques involving face masks, head-only exposures, intratracheal instillation, or other similar techniques that reduce or preclude added dermal and oral exposures. Some alternative techniques are described by Phalen (1976). When alternative techniques are used, the procedures and results should be reported in a manner similar to that required with the use of whole-body inhalation chambers.

(ii) Animals should be tested in a dynamic air flow exposure chamber. The chamber design should be chosen to enable production of an evenly distributed exposure atmosphere throughout the chamber. The chamber design should also minimize crowding of the test animals and maximize their exposure to the test substance by the inhalation route.

(8) Operation measurements. The following measurements should be taken with care to avoid major fluctuations in air concentrations or major discrepancies in the oepration of the chambers:

(i) Air flow. The rate of air flow through the chamber should be measured continuously;

(ii) Chamber concentrations.

(A) Nominal concentrations of organisms should be calculated for each run by dividing the amount of the test substance containing known numbers of organisms per unit volume of the test substance used for the generating system by the air flowing through the chamber during the exposure.

(B) Actual chamber concentrations of organisms should be determined by sampling chamber air near the breathing zone of the animals as frequently as is necessary to obtain an averaged integrated external exposure which is representative of the entire exposure period. The system used to generate the aerosol should be such that the chamber concentrations and particle size distributions are controlled under stable conditions, reflecting the current state-of-the-art, and should not vary in a range greater than 30 percent of the average (range/mean equal to or less than 30 percent);

(iii) Temperature and humidity. The temperature should be maintained at 24 ± 2° C, and the humidity within the chamber at 40-60 percent. Both should be monitored continuously;

(iv) Oxygen. The rate of air flow through the chamber should be adjusted to insure that the oxygen content of the exposure atmosphere is at least 19 percent; and

(v) Particle size measurements. (A) General. The particle size created by the disseminating device must be in the respirable range for the species under test. Aerosol particle size measurements should be made on samples taken at the breathing level of the animals. These analyses should be carried out using techniques and equipment reflective of the state-of-the-art. All of the suspended aerosol (on a gravimetric basis) should be accounted for, even when most of the aerosol is not respirable.

(B) Sizing analysis. The sizing analysis should be in terms of equivalent aerodynamic diameters and should be represented as geometric mean (median) diameters and their geometric standard deviations (see NIOSH syllabus in Appendix), as calculated from log probability graphs or computer programs. The size analyses should be carried out frequently during the development of the generating system to ensure proper stability of aerosol particles, and only as often thereafter during the exposure as is necessary to determine adequately the consistency of particle distributions to which the animals are exposed, maintaining at least 20 percent of the particles at 10 microns or less except when creation of particles in this size range would kill or injure the microorganism under test. At a minimum, these analyses should be carried out twice per hour for liquid test substances, and 4 times per hour for dusts and powders.

(9) Observation. The animals should be observed frequently during the day of dosing and checked at least once each morning and late afternoon for at least 14 days or until all signs of reversible infectivity subside, whichever occurs later. The following should be observed even though the animals recover completely from the exposure: nature and onset of all gross or visible clinical signs of illness such as elevated temperatures, unkempt appearance, altered feeding habits, weight loss, various forms of distress and depression, and similar expressions. In addition, an assay for specific antibody production should be performed if test duration exceeds 14 days.

(10) Sacrifice and necropsy. All test animals surviving at the end of the observation period should be sacrificed. All test animals, whether dying during the test or sacrificed, should be subjected to a complete gross necropsy. In addition, microorganism dissemination, replication, and survival in animal tissues, organs, and fluids, and survival in nasal passages, tracheae, bronchi, and lungs should be determined. Samples should be cultured on laboratory surface or other suitable media to provide qualitative and quantitative measurements of survival in the host organism and multiplication of the test microbial agent.

(c) Data reporting and evaluation. In addition to the requirements in § 80-4 of Subdivision F, the test report should include the following data:

(1) Particulate size;

(2) Description of the chamber design and operation, including chamber type, dimensions, source of make-up air and its conditioning (heating or cooling) for use in the chamber, treatment of exhausted air, housing and maintenance of the animals in the chambers, and similar related information. Equipment for measuring temperature and humidity, the generating system, and the methods of analyzing airborne concentrations of microbial agent and particle sizing should be described;

(3) The following operation data should be tabulated both individually and in summary form, using means and standard deviations (with or without ranges) in tabular form. The data summaries should be grouped according to experimental groups, and differences (such as in temperature and airflow) should be tested for statistical significance.

(i) Airflow rates through the chamber;

(ii) Chamber temperature and humidity;

(iii) Nominal concentrations of microbial agent;

(iv) Actual concentrations of microbial agent; and

(v) Median particle sizes and their geometric standard deviations, and the percentage of particles under 10 microns;

(4) Tabulation of response data (number of animals dying per number of animals exhibiting signs of infectivity per number of animals exposed) at each exposure level for each sex, and the time of death after dosing;

(5) Tabulation of body weights at the beginning of the study and at each 7-day interval thereafter;

(6) The LC50 or LD50 of the microbial agent (calculated for an exposure of one hour or from lethality data) for each sex and each test substance;

(7) Specification of the method used for LC50 or LD50 calculation;

(8) The 95 percent confidence interval for the LC50 or LD50;

(9) The dose-response curve and slope (with confidence limits);

(10) The findings from a histopathological study, if conducted, including a complete record of lesions and abnormalities observed, and the histological characterization of each kind of lesion or abnormality observed, naming those which apparently caused death or moribundity;

(11) The gross pathological findings; and

(12) Evidence of microorganism dissemination, replication, and survival in animal tissues, organs, and fluids, particularly in nasal passages, tracheae, bronchi, and lungs.

(13) Results of the assay for specific antibody production shall be reported in tests exceeding 14 days duration.

(d) Tier progression.

(1) No further testing involving the respiratory route of exposure using bacteria or fungi is necessary.

(2) If evidence of infectivity, organism persistence or replication, or toxicity is observed following the inhalation infectivity/toxicity study using viruses or protozoa is found, then a Tier II acute inhalation study (§ 152-41) with the microbial agent shall be required by 40 CFR § 158.165. Tier II tests on protozoa will use a different species than that used in Tier I and tests on viruses will use newly weaned mice or newly weaned hamsters. In addition, the teratogenicity study (§ 152-47) shall be required for viruses as specified in 40 CFR § 158.165.

(3) If no evidence of acute inhalation infectivity, persistence, replication, or toxicity is observed, then additional testing is not necessary.

(e) References. The following texts and articles give general information as well as sufficient detail to develop and carry out inhalation toxicity/infectivity studies.

(1) General references.

(i) Altman, P.L., et al. 1958. Handbook of Respiration. Aero Medical Laboratory. Wright-Patterson Air Force Base, Ohio. (Includes extensive data on humans and animals.)

(ii) Casarett, L.J., and J. Doull. 1975. Toxicology: Basic Science of Poisons. MacMillan Publishing Co., Inc. New York. (Good text on general toxicology, includes Chapter 9, "Toxicology of the Respiratory System".)

(iii) Comroe, J.H. 1974. Physiology and Respiration. 2nd Ed. Year Book Medical Pub. Chicago. (Includes the anatomy, physiology, physiological testing, and pathology of the lungs in humans.)

(iv) Hatch, T.G., and P. Gross. 1964. Pulmonary Deposition and Retention of Inhaled Aerosol. Academic Press. New York. (Comprehensive text which deals with the anatomy, physiology, deposition and retention, and pathological changes in the lung.)

(v) ICRP Committee, P. Morrow (Chairman). 1966 and 1967. Deposition and retention models for internal dosimetry of the human respiratory tract. Health Phys. 12:173-207 (1966). Errata and revisions to report, Health Phys. 13:1251. (1967).

(vi) Ignoffo, C.M. 1973. Effects of entomopathogens on vertebrates. Ann. N.Y. Acad. Sci. 217:141-172.

(2) Advanced Monographs.

(i) Mercer, T.T., P.E. Morrow, and W. Stober. 1972. Assessment of Airborne Particles. Charles C. Thomas. Springfield, Ill.

(ii) Walton, W.H., ed. 1970. Inhaled Particles III. Proceedings of an International Symposium organized by the British Occupational Hygiene Society. London. September 14-23, 1979. Volumes I and II. Unwin Brothers Limited. Gresham Press. Surrey, England.

(iii) Walton, W.H., ed. 1975. Inhaled Particles. IV. Proceedings of an International Symposium organized by the British Occupational Hygiene Society. London. September 22-26, 1975. Volumes I and II. Pergamon Press, New York.

(3) Exposure Systems.

(i) Drew, R.T., and S. Laskin. 1973. Environmental Inhalation Chambers. Pp. 1-41 in Methods of Animal Experimentation, Vol. IV. Academic Press Inc., New York and London.

(ii) Fraser, D.A., R.E. Bales, M. Lippmann, and H.E. Stokinger. 1959. Exposure Chambers for Research in Animal Inhalation. Public Health Service Monograph No. 57. U.S. Government Printing Office. Washington, D.C.

(iii) Hinners, R.G., J.K. Burkart, and C.L. Punte. 1968. Animal inhalation exposure chambers. *Arch. Environ. Health* 16:194-206.

(iv) Phalen, R.F. 1976. Inhalation exposure of animals. *Environ. Health Perspect*. 16:17-24.

(v) Roe, F.J.C. 1958. Inhalation tests; in Modern Trends in Toxicollogy, Vol. 1. Boyland, E. and R. Goulding, eds. Appleton-Century-Crofts, New York.

(4) *Generating Systems*. Gas and vapor generation is relatively simple compared to the large number of different systems needed to generate aerosols of solids and liquids. Besides the following references, all of the advanced monographs in paragraph (3) contain descriptions of generating systems.

(i) Drew, R.T., and M. Lippman. 1972. Section I. Calibration of Air Sampling Instruments. II. Production of Test Atmospheres for Instrument Calibration; in Air Sampling Instruments. 4th Ed. Am. Conf. Gov. Ind. Hygienists.

(ii) Fraser, D.A., et. al. 1959. Exposure Chambers for Research in Animal Inhalation. Public Health Monograph No. 57. Supt.Doc., U.S. Gov. Print. Off. Washington, D.C.

(iii) Raabe, O.G. 1970. Generation and Characterization of Aerosols. Pg. 123 in Inhalation Carcinogenesis. USAEF Conf-691001. M.G. Hanna et al. Clearinghouse for Federal Scientific and Technical Information. Springfield, Va.

(5) *Sampling Methods*.

(i) Lippman, M. 1972. Respiratory Dust Sampling. Section G in Air Sampling Instruments. 4th Ed. American Conference of Governmental Hygienists, Cincinnati.

(ii) Morrow, P.E. 1964. Evaluation of inhalation hazard based upon the respirable dust concept and the philosophy and application of selective sampling. *Amer. Ind. Hyg. Assoc.* 25:213-236.

(iii) National Institute of Occupational Health and Safety. 1973. The Industrial Environment - Its Evaluation and Control. Supt.Doc., Gov. Print. Off., Washington, D.C. (Has several good chapters on sampling, and on chemical, instrumental, and physical analyses of atmospheres.)

(iv) Peterson, C.M. 1972. Aerosol Sampling for Particle Size Analyses. Section F - Respiratory dust sampling in Air Sampling Instruments. 4th Ed. American Conference of Governmental Hygienists, Cincinnati.

(v) Preining, O., D. Sheesley, N. Djordevic, et al. 1967. The size distribution of aerosols produced by air blast nebulization. J. Colloid and Interface Sci. 23:3.

(vi) Silverman, L., C.E. Billings, M.W. First, et al. 1971. Particle Size Analysis in Industrial Hygiene. Academic Press, Inc., New York and London. (Sampling size analysis and instrumentation with an emphasis on hygiene and air cleaning.)

(6) Pulmonary function testing. Pulmonary function tests have been widely used in the evaluation of human respiratory function but less widely used in animal research. Such analyses may be required to indicate subtle damage to the pulmonary system.

(i) Alarie, Y., A. Krumm, H. Jennings, R. Haddock, et al. 1971. Distribution of ventilation in cyanomologus monkeys. Arch. Environ. Health 22:633. (Illustrates testing in a primate.)

(ii) Amdur, M.O., and J. Mead. 1958. Mechanics of respiration in unanesthetized guinea pigs. Am. J. Physiol. 192:364. (Illustrates function testing in a rodent.)

(iii) Comroe, J.H., et al. 1962. The Lung, Clinical Physiology and Pulmonary Function Tests. 2d Ed. Year Book Med. Publ., Inc., Chicago. (Provides a discussion of pulmonary testing along with anatomy and physiology of the human respiratory system.)

(iv) Comroe, J.H. 1965. Physiology of Respiration. Year Book Med. Publ., Inc., Chicago. [Similiar to Comroe et al., 1962 (above).]

(v) Mauderly, J., and J. Pickrell. 1973. Pulmonary Function Testing of Unanaesthetized Beagle Dogs; in Research Animals in Medicine. L. Harmison, ed. DHEW Pub. No. NIH 72-333. (Illustrates function testing in the dog.)

§ 152-33 Intravenous, intracerebral, and intraperitoneal toxicity/infectivity studies with microbial pest control agents: Tier I.

(a) When required. Data from the following tests are required by 40 CFR 158.165 to support the registration of each manufacturing-use product and each end-use microbial pest control agent as follows:

(1) Intravenous ("IV") infectivity study for bacterial and viral agents;

(2) Intracerebral ("IC") infectivity study for viral and protozoan agents; and

(3) Intraperitoneal ("IP") infectivity study for fungal and protozoan agents.

(b) Test standards. In addition to the general standards set forth in § 150-3 of this subdivision and § 80-3 of Subdivision F, studies outlined in this section should meet the following standards:

(1) Substance to be tested. The technical grade of each active ingredient [termed purest infective form ("PIF") for viruses] shall be tested.

(2) Species. Testing for studies required by this section should be performed with the following test animals:

(i) Newly weaned mouse and newly weaned hamster for IV study with bacteria and viruses;

(ii) Mouse and one other species for IP study with fungi;

(iii) Newborn mouse and newborn hamster for IC study with viruses; and (iv) Mouse and rabbit for IC and IP studies with protozoa.

(3) Sex. Approximately equal numbers of males and females should be used.

(4) Immunodepression. One half of the animals used in these studies should be immunodepressed.

(5) Number of animals. An adequate number of animals should be injected with a single high dose of test substance to permit periodic sacrifice at 1, 2, 3, and 4 weeks (termination of study) post-treatment. At least 5 animals per sex, species, and state of immunodepression per sacrifice for the hamsters and mice should be used.

(6) Control animals. A control group of animals (one half immunodepressed) should be injected with the test vehicle containing killed test organism. All control animals should be sacrificed at four weeks post-treatment. At least five animals per sex, species, and state of immunodepression should be used.

(7) Conduct of test.

(i) Application. Approximately 0.05 ml (for mice and for newborn and newly weaned hamsters) or 0.1 ml (for other animals) of test substance should be injected in accordance with standard procedures and sites for IV, IC, and IP tests. The test substance should be injected in undiluted form. If the test substance is a solid, it should be dissolved in a minimal amount of physiological saline.

(ii) Duration of observation. Surviving animals should be observed for at least four weeks after dosing, or until all signs of reversible infectivity in survivors subside, whichever occurs later.

(iii) Observations. The animals should be observed frequently during the day of dosing and checked at least once each morning and late afternoon thereafter. The following should be observed even if the animals recover completely from the exposure: nature and onset of all gross or visible clinical signs of illness such as unkempt appearance, altered feeding habits, weight loss, physical depression, depression, and similar expressions.

(iv) Assay for production of specific antibodies. An assay for the production of specific antibodies should be performed in each study, excepting those using newborn or newly weaned test animals.

(v) Sacrifice and necropsy. All test animals surviving at the end of the observation period should be sacrificed. All test animals, whether dying during the test or sacrificed, should be subjected to a complete necropsy. In addition to reporting gross pathology, evidence of organism survival and/or multiplication in blood and at distant sites such as the spleen, liver, lung, and brain should be determined by culturing tissues on laboratory or other suitable media or host to provide qualitative and quantitative evidence of possible survival and multiplication of the test organism(s).

(8) Dose quantification. Titers of microbial preparations administered to test animals should be determined by plating dilutions on suitable media or host organisms for enumeration of viable organisms.

(c) Data reporting and evaluation. In addition to the information required by § 80-4 of Subdivision F, the test report should include the following information:

(1) Tabulation of response data by species, sex, and state of immunodepression as to numbers of animals exhibiting signs of infectivity and/or confirmed recovery of test animal per number of animals exposed;

(2) The time of death after dosing;

(3) Results of gross pathological examinations, including recovery adn approximate numbers of viable test microorganisms found in various cultured tissues; and

(4) Identification of the test microorganism, including:

(i) Genus, species, serotype and strain (to the extent possible) according to current acceptable taxonomy; and

(ii) The percent of unknown fermentation solids or other materials present to account for 100 percent of the sample tested.

(5) Results of the assay for production of specific antibodies when applicable.

(d) Tier progression.

(1) If evidence of prolonged survival or replication in mammalian hosts and significant damage to mammalian cells is observed when viruses are tested, then data on teratogenicity testing (§ 152-47) are required by 40 CFR 158.165.

(2) If evidence of infectivity or organism persistence, replication, or toxicity is observed when protozoa are tested, then additional intraperitoneal and intracerebral testing (§ 152-43) and acute oral testing (§ 152-40) is required by 40 CFR 158.165.

(3) If evidence of infectivity is observed (e.g., prolonged survival and/or replication) when bacteria or fungi are tested, then the virulence enhancement test in Tier II (§ 152-48) using the mouse or hamster shall be required by 40 CFR 158.165.

(4) If no evidence of infectivity or prolonged survival is observed, then further testing is not necessary.

(e) References. The following references contain useful information for developing an acceptable protocol.

(1) Hansen, G.D. 1973. Elimination rate of Bacillus thuringiensis spores administered to mice. Abbott Laboratories T-9, Project no. 70111. Information supplied to the subcommittee by Abbott Laboratories, North Chicago, Ill.

(2) Ignoffo, C.M. 1973. Effects of entomopathogens on vertebrates. Ann. N.Y. Acad. Sci. 217:141-164.

(3) Lamanna, C., and L. Jones. 1963. Lethality for mice of vegetative and spore forms of Bacillus cereus and Bacillus cereus-like insect pathogens injected intraperitoneally and subcutaneously. J. Bacteriol. 85: 532-535.

(4) Leise, J.M., C.H. Carter, H. Friedlander, and S.W. Freed. 1959. Criteria for the identification of Bacillus anthracis. J. Bacteriol.77: 655-660.

§ 152-34 Primary dermal irritation study with microbial pest control agents: Tier I.

(a) When required. Data on primary dermal irritation are required by 40 CFR § 158.165 to support the registration of each manufacturing-use product and each end-use product.

(b) Test standards. The general standards set forth in § 150-3 of this subdivision and § 80-3 of Subdivision F should apply. In addition to these general test standards, a primary dermal irritation study should meet the following standards:

(1) Substances to be tested.

(i) The manufacturing-use product shall be tested to support the registration of a manufacturinguse product.

(ii) The end-use product shall be tested to support the registration of an end-use product.

(2) Species and age. Testing should be performed with young adult guinea pigs or rabbits.

(3) Condition of test substances.

(i) If the substance is a liquid, it should be applied undiluted.

(ii) If the test substance is a solid, it should be slightly moistened with physiological saline before application.

(4) Number of animals. At least six animals shall be used.

(5) Number and selection of dose levels. A dose of 0.5 ml of liquid or 0.5 g of solid or semi-solid microbial preparation is to be applied to each application site.

(6) Dose quantification. Titers of microbial suspension administered to test animals should be performed by plating dilutions on laboratory surface media or other suitable media or host organism for enumeration of viable organisms.

(7) Control groups.

(i) A vehicle control group is recommended if the vehicle is known to cause any toxic dermal reactions or if there is insufficient information concerning the dermal effects of the vehicle.

(ii) Separate animals are not necessary for an untreated control group. Each animal serves as its own control.

(8) Conduct of test. The test substance is introduced under one-inch square gauze patches. The patches should be applied to two intact and two abraded skin sites on each animal. In all animals, the application sites should be clipped free of hair. The abrasion should penetrate the stratum corneum, but not the dermis. A wrapping material such as gauze covered by an impervious, non-reactive rubberized or plastic material should be used to retard evaporation and to keep the test substance in contact with the skin. The animals should be restrained. The test substance must be kept in contact with the skin for 24 hours. At the end of the exposure period, the wrapping should be removed and the skin wiped (but not washed) to remove any test substance still remaining. It may be necessary to rinse off the material if colored test substances are used.

(9) Observation and scoring. Animals shall be observed and signs of erythema and edema shall be scored at 24 hours and 72 hours after application of the test substance. The irritation is to be scored according to the technique of J.H. Draize (1959). Observation for irritation and scoring of any irritation shall continue daily until all irritation subsides or is obviously irreversible.

(c) Data reporting and evaluation. In addition to the applicable general information required by § 80-4 of Subdivision F [excepting paragraphs (b)(2)(iii)(A) and (b)(2)(vii)], the test report shall include the following information:

(1) In tabular form, the following data for each individual animal and averages and ranges for each test group:

(i) Scores for erythema and edema at 24 hours, at 72 hours, and at any subsequent observation, and;

(ii) Primary skin irritation scores according to the technique of Draize.

(d) Tier progression.

(1) No further testing is necessary for manufacturing-use products.

(2) If evidence of primary dermal irritation is observed (marked edema or broad erythema) in tests conducted on the end-use formulated product, then:

(i) Primary dermal irritation studies in the guinea pig shall be required by 40 CFR 158.165 using use dilutions of the end-use product (§ 152-44).

(3) If no evidence of primary dermal irritation is observed, then further testing is not necessary.

§ 152-35 Primary eye irritation study with microbial pest control agents: Tier I.

(a) When required. Data on primary eye irritation are required by 40 CFR 158.165 to support the registration of each manufacturing-use product and each end-use product.

(b) Test standards. The general standards set forth in § 80-3 of Subdivision F apply. In addition to these general test standards, a primary eye irritation study should meet the following standards:

(1) Substances to be tested.

(i) The manufacturing-use product shall be tested to support the registration of a manufacturinguse product.

(ii) The end-use product shall be tested to support the registration of an end-use product.

(2) Species and age. Testing should be performed using young adult rabbits.

(3) Condition of test substance.

(i) If the test substance is in liquid form, it should be applied undiluted.

(ii) If the test substance is a solid, it should be slightly moistened with physiological saline before application.

(4) Numbers of animals. At least nine animals should be used.

(5) Number and selection of dose. A dose of 0.1 ml of liquid or 100 mg of solid should normally be applied to each test eye. Smaller quantities may be used when the standard quantities would be lethal, or when 100 mg of the solid cannot feasibly be administered to the eye.

(6) Dose quantification. Titers of microbial suspensions to test animals should be performed by plating dilutions on laboratory surface or other suitable media or a host organism for enumeration of viable organisms.

(7) Caging. Caging should be designed to minimize exposure to sawdust, wood chips, and other extraneous materials that might enter the eye.

(8) Conduct of test. The test substance should be placed on the everted lower lid of one eye; the upper and lower lids are then then to be gently held together for 1 second before releasing to prevent loss of material. The other eye, remaining untreated, serves as a control. The treated eyes of 6 rabbits shall remain unwashed. The remaining 3 rabbits shall receive test material, and then the treated eye should be flushed for one minute with lukewarm water starting no sooner than 20-30 seconds after instillation. A local anaesthetic to reduce pain in test animals may be used prior to administration of the test substance, provided that evidence can be presented indicating no significant difference in toxic reaction to the test substance will result from use of the anaesthetic.

(9) Observation and scoring.

(i) Observation. Readings of ocular lesions should be made at 24, 48, and 72 hours after treatment and at 4 and 7 days after treatment. Readings should be made every 3 days thereafter, if injury persists, for at least 21 days after treatment or until all signs of reversible toxicity subside. Grading and scoring of irritation are to be performed in accordance with Table 5 [from Draize et al. (1965)]. The most serious effects, such as pannus or blistering of the conjunctivae and other effects indicative of corrosive action should be reported separately.

(c) Data reporting and evaluation. In addition to the applicable general information required by § 80-4 of Subdivision F, the test report should include, in tabular form, the following data for each individual animal and the averages and range for each test group (eyes washed and unwashed):

(1) The primary eye irritation score at 24, 48, and 72 hours and 4, 7, and 21 days and any other readings; and

(2) A description of any serious lesions.

(d) Tier progression.

(1) No further testing is necessary for manufacturing-use products.

(2) If evidence of primary ocular irritation (e.g., severe ocular lesions) is observed in tests conducted on the end-use formulated product, then primary ocular irritation studies in the rabbit (§ 155-45) shall be required by 40 CFR 158.165 using use-dilutions of the end-use formulated product.

(3) If no evidence of primary ocular irritation is observed, then further testing is not necessary.

Table 5--WEIGHTED SCORES FOR GRADING THE SEVERITY OF OCULAR LESIONS

I. Cornea

(A) Opacity - degree of density (area taken for reading)

- Scattered or diffuse area, details of iris clearly visible.	1
- Easily discernible translucent areas, details of iris slightly obscured	2
- Opalescent areas, no details of iris visible, size of pupil barely discernible	3
- Opaque, iris invisible.	4

(B) Area of cornea involved

- One quarter (or less) but not zero.	1
- Greater than one quarter but less than one-half	2
- Greater than one-half but less than three quarters	3
- Greater than three quarters	4

Score equals A x B x 5 Total maximum = 80

II. Iris

(A) Values

- Folds above normal, congestion, swelling, circumcorneal injection (any one or all of these or combination of any thereof), iris still reacting to light (sluggish reaction is positive)	1
- No reaction to light, hemorrhage; gross destruction (any one or all of these)	2

Score equals A x 5 Total possible maximum = 10

III. Conjunctivae

(A) Redness (refers to palpebral conjunctivae only)

- Vessels definitely injected above normal.	1
- More diffuse, deeper crimson red, individual vessels not easily discernible	2
- Diffuse beefy red	3

(B) Chemosis

- Any swelling greater than normal (includes nictitation membrane)	1
- Obvious swelling with partial eversion of the lids	2
- Swelling with lids about half closed.	3
- Swelling with lids about half closed to completely closed	4

(C) Discharge

- Any amount different from normal (does not include small amount observed in inner canthus of normal animals)	1
- Discharge with moistening of the lids and hairs just adjacent to the lids	2
- Discharge with moistening of the lids and considerable area around the eye	3

Score equals (A + B + C) x 2 Total possible maximum = 20

The maximum total score is the sum of all scores obtained for the cornea, iris, and conjunctivae.

§ 152-36 Hypersensitivity study with microbial pest control agents: Tier I.

(a) When required. Data from a hypersensitivity study are required by 40 CFR § 158.165 to support the registration of each end-use product for which commonly recognized use practices will result in repeated human contact by inhalation or dermal routes and each manufacturing-use product which legally may be used to formulate such an end-use product.

(b) Test standards. In addition to the applicable general standards set forth in § 150-3 of this subdivision and § 80-3 of Subdivision F, a hypersensitivity study shall meet the following standards.

(1) Substance to be tested.

(i) The manufacturing-use product shall be tested to support the registration of a manufacturing-use product.

(ii) The end-use product shall be tested to support the registration of an end-use product.

(2) Condition of test substance. The test substance should be applied undiluted. If the test substance causes marked irritation, it should be diluted with physiological saline until a concentration is found which produces only slight irritation. If the test substance is a solid to be injected intradermally, it should be dissolved in a minimum amount of physiological saline.

(3) Species. The test should be performed in the hamster or guinea pig.

(4) Age and sex. Young adult males should be used when albino guinea pigs are tested. Young adults of either sex may be used when hamsters are tested.

(5) Number of animals. At least 10 animals should be used.

(6) Dose quantification. Titers of microbial suspensions should be determined by plating dilutions on laboratory surface or other suitable media or a host organism for enumeration of viable organisms.

(7) Number and selection of dose levels. (i) An initial dose of 0.05 ml should be injected intradermally. This dose shall be followed by injection of 0.1 ml three times weekly on alternate days for three weeks, so that a total of ten treatments is administered. Two weeks after the tenth sensitizing treatment, the animals should be challenged by a final injection (Landsteiner and Jacobs, 1935);

(8) Controls. (i) A positive control, using a known sensitizing agent, is recommended; and

(ii) A concurrent vehicle control group is not required.

(9) Conduct of test.

(1) Preparation of test animals. Hair is removed first by clipping and then by shaving to form a strip running from flank to trunk along each side of each animal. This procedure should be repeated as necessary.

(ii) Intradermal injection. After preparation of the test animal, the test substance is injected intradermally. The first sensitizing injection is be made at one end of one strip. The succeeding injections should be made by moving along the shaved strip choosing a new location for each treatment.

(10) Observation and scoring. Erythema, edema, and other lesions are scored at 24 hours and 48 hours following each application, according to the standard method (Draize, 1959).

(c) Data reporting and evaluation. In addition to the applicable basic information in § 80-4 of Subpart F, the following information should be reported:

(1) Tabulated scores for each animal for erythema and edema at 24 and 48 hours post-application or post-injection; and

(2) Tabulated average scores from all sensitizing treatments, and the score of the challenge treatment.

(d) Tier progression. No tier progression from the hypersensitivity study is necessary.

§ 152-37 Hypersensitivity incidents with microbial pest control agents: Tier I.

(a) When required. Data on incidents of hypersensitivity of humans or domestic animals that occur during the production or testing of the technical grade of the active ingredient, the manufacturing-use product, or the end-use product should be reported with the toxicology data supplied in support of an application for registration. For reporting of incidents taking place after registration, refer to the requirements in connection with sec. 6(a)(2) of FIFRA.

(b) Reporting. The reporting requirements for these incidents should be the same as those for conventional chemical pesticide incident reports as specified in the Pesticide Incident Report form (EPA form number 8550-5, OMB form number 158-R0008). The following information should be provided if available:

(1) The name of the microbial agent;

(2) The length of exposure to the agent;

(3) The time, date and location of exposure to the agent;

(4) The situation or circumstances under which exposure to the agent occurred; and

(5) Any clinical observations.

§ 152-38 Effects of microbial pest control agents on the cellular immune response system: Tier I.

(a) Test description. The following tests of effects on immunocompetence are included in the cellular immune response studies with microbial pest control agents:

(1) Blood cell count;

(2) Leukocyte response (B and T cells);

(3) Total leukocyte number (T and B cell ratios);

(4) Macrophage number and function; and

(5) Serum protein determination.

(b) When required. Data on cellular immune response studies are required by 40 CFR § 158.165 to support the registration of each end-use product and each manufacturing use product. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(c) Test standards. In addition to the test standards set forth in § 150-3 of this subdivision, the following standards should be met:

(1) Test substance. The technical grade of the microbial agent shall be tested.

(2) Dosage. At least three doses of a given microbial agent should be separately administered to test mice prior to performing the cellular immune response evaluations.

(3) Test methods. The test methods for the five different cellular immune response tests described in (i) through (v) apply for any candidate microbial agent (i.e., bacterial, fungi, virus, or protozoa):

(i) Blood cell count. Three groups of 10 male and 10 female mice each are separately injected (intraperitoneally) with a 0.5 ml dose containing different concentrations of test substance. One group of mice are injected with undiluted test substance, one with test substance diluted 10x, and a third group injected with agent diluted 100x. Three groups of five mice each injected with physiological saline should serve as controls. Routine blood counts should be performed at 15 and 30 days after exposure. Standard hemacytometer assays should be utilized to determine the total number of peripheral blood leukocytes as well as differential counts.

(ii) Leukocyte response (T and B cells). Three groups of treated mice and three groups of control mice are prepared as described above (i) for blood cell counts. Fifteen and 30 days after treatment, treated and control mice are to be tested for absolute peripheral blood T and B lymphocyte numbers, and/or their ratio, in order to ascertain whether or not there has been a significant shift in the populations of these cells. An immunofluorescent antibody test should be used to determine the number of peripheral blood leukocytes exhibiting abnormal numbers of total surface immunoglobulins. The E-rosette assay with sheep erythrocytes should be used as a standard technique for ascertaining the number of T cells in the peripheral blood of mice.

(iii) Total leukocyte number (T and B cell ratios). Three groups of treated mice and three groups of control mice are prepared as described above (i) for blood cell counts. At several time intervals after treatment, such as 15 and 30 days, treated and control mice shall be tested for absolute peripheral blood B and T lymphocyte number and/or their ratio in order to ascertain whether there has been a significant shift in the population of these cells. An immunofluorescent antibody test using antimouse immunoglobulin serum is a standard method and could be used to determine the number of peripheral blood leukocytes which exhibit changes in surface immunoglobulins characteristic for B lymphocytes. For the T cells, indirect immunofluorescence assays using standard techniques should also be used, as in § 152-18(c)(4)(ii) and (iii). (See references for cellular immune response test § 152-18.)

(iv) Macrophage number and function. One group of at least 5 mice are each injected intraperitoneally with 0.5 ml of undiluted test substance. Fifteen and 30 days after exposure, the number and percent of peritoneal and splenic macrophages should be determined by standard phagocytic index tests, such as the uptake of latex diffusion assays and electrophoresis. Amounts of individual classes of immunoglobulins may be determined by immunoelectrophoresis to determine levels of Ig G, M, A, and D.

(v) Serum protein determination. Five mice should be injected with 0.5 ml of test substance. Seven and 15 days after exposure, serum protein and immunoglobulin levels should be determined by standard radial-gel diffusion assays and electrophoresis. Amounts of individual classes of immunoglobulins may be determined by immunoelectrophoresis to determine levels of Ig G, M, A, and D.

(4) Dose quantification. Determination of the number of organisms in microbial suspensions administered to test animals should be performed by plating dilutions on laboratory media or host organism for enumeration of viable organisms.

(d) Tier progression. (1) If any indication of abnormality is observed in any of the tests specified in paragraph (a) of this section, Tier II cellular immune response studies (§ 152-46) are required by 40 CFR 158.165.

(2) If no indication of abnormality is observed in the tests conducted as specified in paragraph (a) of this section, then no further testing is necessary.

(e) References. Refer to § 152-18(f).

§ 152-39 Tissue culture tests with viral agents: Tier I.

(a) When required. Data from tissue culture tests (with viral agents) are required by 40 CFR § 158.165 to support the registration of each manufacturing-use product and each end-use product. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Test standards. (1) Substance to be tested. The purest, most concentrated and infectious form of the virus shall be used. Virus preparations should be free of insect hemolymph, as this may prove toxic to cells in culture. The inoculum should be titered by the most sensitive assay available and in the most permissive host system (cell culture or, if not available, host insect). For testing in the model systems, a minimum of five plaque-forming units per cell is required when a plaque assay for the virus is available, or seven LD50's per cell when a plaque assay for the virus is not available. If not feasible, the reasons for lack of compliance should be noted and submitted, and maximal amounts of infectious virus compatible with tissue culture systems shall be assayed.

(2) Cell cultures. The following cells should be used: 1 human line, e.g., WI38; 1 human primary cell type, e.g., foreskin; 1 human continuous line, e.g., HeLa; 1 primate continuous line, e.g., monkey CV-1 or BSC-1 and, for assessment of possible slowly developing virus-cell interactions that could lead to virus persistence and/or malignant transformation, mouse 3T3 cells.

(3) Number of test systems. Replicate cultures containing a minimum of 10^5 cells each should be inoculated in each test.

(4) Observation of gross morphological changes (cytopathic effects). Two confluent plates should be examined daily by light microscopy for a minimum of 14 days for human and primate lines, and weekly intervals for up to one month for the 3T3 cells.

(5) Inhibition of cell division. Two sub-confluent plates should be used for measuring the ability of infected cells to grow to confluency after inoculation.

(6) Bioassay of culture fluid. Cells and culture fluid from replicate confluent plates should be assayed for infectious virus on alternate days for 14 days after inoculation. The bioassay chosen should be the most sensitive available for detecting infectious virus.

(7) Assay for decay of input virus and potential appearance of viral proteins and nucleic acid. (i) A sensitive quantitative immunological test (e.g., the enzyme-linked immunosorbent assay) should be performed on replicate cultures on alternate days for 14 days.

(ii) A sensitive quantitative molecular hybridization test for viral nucleic acid (e.g., kinetics of reassociation of highly labeled viral DNA probe using infected cell DNA as driver) on replicate cultures on alternate days for 14 days should be performed.

(8) Controls. In each instance, mock-infected cultures should be similarly analyzed. For each series of tests the inoculum will be tested in the permissive cell line or insect host as a positive control and for direct reference to the data obtained from the vertebrate cell lines.

(c) Data reporting and evaluation. The following information should be provided on each test:

(1) Cytopathic effects (CPE) in the cell monolayers.

(i) The appearance of CPE in the cell monolayers described to differentiate between cell destruction induced by the test virus and nonspecific effects.

(ii) Results of tube culture inspection for microscopic evidence of cytopathic effects recorded as:

1+ = suggestive of virus-induced morphologic changes;
2+ = definite morphologic changes;
3+ = more than 50% cell degeneration; or
4+ = complete cell destruction.

(iii) The $TCID_{50}$ value calculated by a statistical method (i.e., the Reed and Meunch Method). For computation of the infectivity results, only tubes showing a 2+ CPE or greater are considered to be infected.

(2) Inhibition of cell division.

(i) The procedure used for the study of cell-division inhibition should be detailed.

(ii) The initial cell-number seeded for adequate proliferation determined by either hemocytometer counting or electronic enumeration.

(iii) Results of the cell-number of infected and control cultures monitored during the of study, expressed as a percentage as follows:

$$\frac{\text{Average cell count, infected cultures - initial cell count}}{\text{Average cell count, control cultures - initial cell count}} \text{ X } 100$$

(iv) The percentage of confluency of cell monolayers from infected and control cultures (determined after an appropriate period of incubation).

(v) Any evidence of mitotic process prevention or interference with chromosomal replication.

(3) Bioassay of culture fluid.

(i) Susceptible host sytem used for viral detection.

(ii) Records of the dilutions in the virus assay (e.g., 10^{-1}, 10^{-2}). The serial dilution method is required.

(iii) The percentage infectivity calculated from the number of infected host cells following inoculation with an appropriate series of consecutive virus dilution.

(4) Data from an assay for decay of input virus.

(i) Identity of viral antigens that do not grow well in cell culture.

(A) Details of procedures of the Enzyme-Linked Immunosorbent Assay (ELISA) for viral detection.

(ii) Report the cellular location of viral antigens in the infected cell culture.

(A) Details of procedures of the immunofluorescence technique.

(5) Assay for potential appearance of viral proteins and nucleic acid.

(i) Details of procedures of the DNA-DNA reassociation kinetics.

(d) Tier progression.

(1) If tissue culture tests show evidence of replication or persistence of the viral agent, or damage to host cells, then additional testing in Tier II is required by 40 CFR 158.165. The specific Tier II test requirements will depend on the results of the tissue culture test and will be determined after consultation with the Agency.

(2) If tissue culture tests show no evidence of replication or persistence of the viral agent, or damage to host cells, then no further testing is necessary.

(e) References.

(1) Bullock, S.L., and K.W. Walls. 1977. Evaluation of some of the parameters of the enzyme-linked immunospecific assay. J. Infect. Dis. 136: S279-S285.

(2) Engvall, E., and P. Perlmann. 1972. Enzyme-linked immunosorbent assay. ELISA. III. Quantification of specific antibodies by enzyme-linked antiglobulin in antigen-coated tubes. J. Immunol. 109:129-135.

(3) Gelb, L.D., D.E. Kohne, and M.A. Martin. 1971. Quantitation of simian virus 40 sequences in African Green monkey, mouse and virustransformed cell genomes. J. Mol. Biol. 57:129-145.

(4) Goldman, M. 1968. Fluorescent Antibody Methods. Academic Press, Inc. New York, N.Y.

(5) Hermann, J.E., R.M. Hendry, and M.F. Collins. 1979. Factors involved in enzyme-linked immunoassay of viruses and evaluation of the method for enteroviruses. J. Clin. Microbiol. 10:210-217.

(6) Rigby, P.W.J., M. Kieckmann, C. Rhodes, and P. Berg. 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. 113:237-251.

(7) Wold, W.S.M., M. Green, and J.K. Mackey. 1978. Methods and rationale for analysis of human tumors for nucleic acid sequences of oncogenic human DNA viruses. Methods Cancer Res. 15:69-161.

(8) Yolken, R.H., H.W. Kim, T. Clem, R.G. Wyatt, A.R. Kalica, R.M. Chanock, and A.Z. Kapikian. 1977. Enzyme-linked immunosorbent assay (ELISA) for detection of human reovirus-like agent of infantile gastroenteritis. Lancet 2:263-266.

Group B-2: Tier II Testing.

§ 152-40 Acute oral toxicity/infectivity studies with viral or protozoan agents: Tier II.

(a) When required.

(1) End-use formulated products. Data on the acute oral infectivity of viral or protozoan agents (Tier II) are required by 40 CFR § 158.165 to support the registration of each end-use product for which survival, replication, infectivity, toxicity, or persistence of the microbial agent (virus or protozoa) is observed in the test animals treated in the Tier I acute oral infectivity tests (§ 152-30) or the intraperitoneal/intracerebral injection test for protozoa (§ 152-33).

(2) Manufacturing-use products. Data on the acute oral infectivity of viral or protozoan agents (Tier II) are required by 40 CFR § 158.165 to support the registration of each manufacturing-use product for which survival, replication, infectivity, toxicity, or persistence of the microbial agent (virus or protozoa) is observed in the test animals treated in the comparable Tier I acute oral infectivity tests (§ 152-30) or the intraperitoneal/intracerebral injection test for protozoa (§ 152-33).

(b) Test standards. In addition to the applicable general standards set forth in § 150-3 of this subdivision and § 80-3 of Subdivision F, the acute oral infectivity test with protozoa or viruses should be conducted in accordance with the standards set forth in § 153-30 of this subdivision, with the following exceptions:

(1) Substance to be tested. (i) The end-use product shall be tested to support the registration of an end-use product.

(ii) The manufacturing-use product shall be tested to support the registration of a manufacturing-use product.

(2) Species. (i) For viral agents, testing should be conducted on newly-weaned mice and newly-weaned hamsters.

(ii) For protozoan agents, testing should be conducted on beagle puppies.

(3) Number of animals and selection of dose levels.

(i) In tests using mice or hamsters, a trial test is recommended for the purpose of establishing a dose level higher than the expected LD50. If data based on testing with at least five animals per sex are submitted showing that the oral LD50 is greater than 5 g/kg, no further testing at other dose levels is necessary. If death occurs, the requirements of paragraph (b)(3)(ii) of this section apply.

(ii) At least three dose levels spaced appropriately should be tested using adequate numbers of animals per dose level to produce test groups with mortality rates between 10 percent and 90 percent to permit the calculation of an LD50 for males and females with a 95 percent confidence interval of 20 percent or less.

(iii) In tests using puppies, doses as large as possible should be administered. Only one dose per animal is administered. Adequate numbers of animals are tested so that necropsies can be conducted at three time periods during the day.

(4) Duration of the test.

(i) Surviving exposed animals and appropriate controls should be observed for at least 60 days or until signs of reversible infectivity subside, whichever occurs later.

(5) Conduct of test. (i) Fasting. Food should be withheld from the animals 24 hours prior to dosing mice with protozoan agents.

(ii) Observation. The animals should be observed frequently during the day of dosing and checked at least once each morning and late afternoon thereafter. The following should be recorded even though the animals recover completely from the exposure: nature and onset of all gross or visible clinical signs of illness such as elevated temperature, unkempt appearance, altered feeding habits, weight loss, and various forms of distress and physical depression.

(iii) Sacrifice and necropsy. All test animals surviving at termination of the observation period should be sacrificed. All test animals, whether dying during the test or sacrificed, should be subjected to a complete gross necropsy. If pathology is observed, organs and tissues must be examined microscopically. In addition to gross pathology, the following should be determined: microorganism dissemination, replication and survival in animal tissues, organs, and fluids, including survival in the intestinal tract. Samples should be cultured on laboratory surface or other suitable media or a host organism to provide qualitative and quantitative measurements of survival and multiplication of the microorganism. In addition, for tests of viral agents, samples of blood, spleen, liver, heart, skeletal muscle, lung, and small intestine should be examined for infectivity. These samples should also be assayed by sensitive molecular techniques, to be performed using radioimmunoassay, enzyme-linked immunosorbant assay, or nucleic acid hybridization tests.

(c) Data reporting and evaluation. In addition to the information required by § 80-4 of Subdivision F and § 152-30 of this subdivision, the test report should include the following information:

(1) When using the serologic and nucleic acid tests to detect viruses, the sensitivity, specificity, and limits of each assay shall be defined by a comparison to a standard assay using the permissive system.

(d) Tier progression. (1) A chronic feeding study (§ 152-50) is required by 40 CFR § 158.165 for viral agents if the potential for chronic adverse effects is indicated, based on:

(i) Results of this study (§ 152-40);

(ii) Results of comparable tests in § 152-30 conducted on other species; and

(iii) The extent of expected human exposure based on residue analysis data developed in accordance with section series 153.

(2) Data on a chronic feeding study (§ 152-50) and on oncogenicity studies (§ 152-51) are required by 40 CFR § 158.165 for protozoa if the potential for oncogenic, or other chronic adverse effects are indicated based on:

(i) Results of this study (§ 152-40);

(ii) Results of comparable tests in § 152-30 conducted on other species; and

(iii) The extent of expected human exposure based on residue analysis data developed in accordance with section series 153.

(3) If agent replication, persistence, and the potential for chronic adverse effects are not indicated, then no further testing is necessary.

(e) References. Refer to § 152-30.

§ 152-41 Acute inhalation toxicity/infectivity study with viral or protozoan agents: Tier II.

(a) When required.

(1) End-use products. Data on acute inhalation (Tier II) are required by 40 CFR § 158.165 to support the registration of each end-use product for which survival, replication, infectivity, toxicity, or persistence of the microbial agent (virus or protozoa) is observed in the test animals treated in the comparable Tier I acute inhalation tests (§ 152-32).

(2) Manufacturing-use products. Data on acute inhalation (Tier II) are required by 40 CFR § 158.165 to support the registration of each manufacturing-use product for which survival, replication, infectivity, toxicity, or persistence of the microbial agent (virus or protozoa) is observed in the test animals treated in the comparable Tier I acute inhalation tests (§ 152-32).

(b) Test standards. In addition to the applicable general standards set forth in § 150-3 of this subdivision and § 80-3 of Subdivision F, the acute inhalation study with viruses or protozoa should be conducted in accordance with the standards set forth in § 152-32 of this subdivision, with the following exceptions:

(1) Substance to be tested.

(i) The end-use product shall be tested to support the registration of an end-use product.

(ii) The manufacturing-use product shall be tested to support the registration of a manufacturing-use product.

(2) Species.

(i) For viral agents, testing should be conducted on newly-weaned mice and newly-weaned hamsters.

(ii) For protozoan agents, testing should be conducted on a different species than used in the Tier I test [§ 152-32(b)(1)].

(3) Duration of test. Test animals should be observed for 60 days, or until signs of reversible infectivity subside, whichever occurs later.

(4) Conduct of test.

(i) Observations. The animals should be observed frequently during the day of dosing and checked at least once each morning and late afternoon thereafter. Even if the animals recover completely from the exposure, the nature and onset of all gross or visible clinical signs of illness such as elevated temperature, unkempt appearance, altered feeding habits, weight loss, various forms of distress, physical depression, and other similar expressions should be recorded.

(ii) Sacrifice and necropsy. All test animals surviving at termination of the observation period should be sacrificed. All test animals, whether dying during the test or sacrificed, should be subjected to a complete gross necropsy. If gross pathology is observed, organs and tissues must be examined microscopically. In addition to reporting gross pathology, microorganism dissemination, replication, and survival in animal tissues, organs, and fluids should be determined, including survival in the intestinal tract. Samples should be cultured on laboratory surface or other suitable media or a host organism to provide qualitative and quantitative measurements of survival and multiplication of the microorganism. In addition, for tests of viral agents, samples of blood, spleen, liver, heart, skeletal muscle, lung, and small intestine should be examined for infectivity and be assayed by sensitive molecular techniques, to be conducted using radioimmunoassay, and/or enzyme-linked immunosorbant assay, and by nucleic acid hybridization tests.

(c) Data reporting and evaluation. In addition to the provisions set forth in § 152-32 of this subdivision, the following apply:

(1) When using serologic and nucleic acid tests to detect virus, the sensitivity, specificity, and limits of each assay should be defined by comparison to a standard assay using the permissive system.

(d) Tier progression.

(1) If replication or persistence in mammalian species is indicated, then Tier III oncogenicity and mutagenicity tests (§§ 152-51 and -52) may be required as specified in 40 CFR § 158.165.

(2) Certain additional studies in Tier III are required by 40 CFR 158.165 if the potential for chronic or other adverse effects is indicated based on:

(i) Results of this study (§ 152-41);

(ii) Results of comparable tests in § 152-32 conducted on other species; and

(iii) The extent of human exposure expected based on residue analysis data developed in accordance with section series 153. Specific test requirements shall be established based on results of this test.

(3) If the potential for chronic or other adverse effects is not indicated, then no further testing is necessasry.

(e) *References*. The following references provide information on virus detection. In addition, refer to § 152-32 for references on conducting inhalation studies.

(1) Bullock, S.L. and K.W. Walls. 1977. Evaluation of some of the parameters of the enzyme-linked immunospecific assay. *J. Infect. Dis.* 136:5279-5285.

(2) Engvall, E. and P. Perlmann. 1972. Enzyme-linked immunosorbent assay. ELISA. III. Quantification of specific antibodies by enzyme-linked antiglobulin in antigen-coated tubes. *J. Immunol.* 109:129-135.

(3) Hermann, J.E., R.M. Hendry, and M.F. Collins. 1979. Factors involved in enzyme-linked immuno-assay of viruses and evaluation of the method for enteroviruses. *J. Clin. Microbiol*. 10:210-217.

(4) Yolken, R. H., H.W. Kim, T. Clem, R.G. Wyatt, A.R. Kalica, R.M. Chanock, and A.Z. Kapikian. 1977. Enzyme-linked immunoabsorbent assay (ELISA) for detection of human reoviruslike agent of infantile gastroenteritis. *Lancet* 2:263-266.

(5) Gelb, L.D., D.E. Kohne, and M.A. Martin. 1971. Quantitation of simian virus 40 sequences in Africa Green monkey, mouse and virustransformed cell genomes. *J. Mol. Biol*. 57:129-145.

(6) Rigby, P.W.J., M. Kiekmann, C. Rhodes, and P. Berg. 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. *J. Mol. Biol.* 113:237-251.

(7) Wold, W.S.M., M. Green, and J.K. Mackey. 1979. Methods and rationale for analysis of human tumors for nucleic acid sequences of oncogenic human DNA viruses. Methods Cancer Res., Vol. 15.

§ 152-42 Subchronic oral dosing study with protozoa: Tier II.

(a) When required.

Data from subchronic oral infectivity tests are required to support the registration of each end-use product for which there is evidence of survival, replication, infectivity, or persistence of the protozoan agent in the Tier I oral infectivity test (§ 152-30) and to support each manufacturing-use product which may legally be used to formulate such an end-use product. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Test standards. The applicable general test standards and applicable specific test standards set forth in § 80-3 and 82(e) through (g), respectively, of Subdivision F shall apply, with the following exceptions:

(1) Species. Testing should be performed in one mammalian species, the mouse, rat, or dog.

(2) Observation of animals. In addition to the applicable specifications set forth in § 82-1 of Subdivision F, the specifications set forth in § 152-40 of this subdivision apply.

(3) Sacrifice and necropsy. In addition to the applicable specifications set forth in § 82-1 of Subdivision F, the specifications set forth in § 152-40 of this subdivision shall apply.

(c) Data reporting and evaluation. The applicable provisions set forth in § 82-1 of Subdivision F and § 152-40 of this subdivision apply.

(d) Tier progression.

(1) Data on oncogenicity (§ 152-51), mutagenicity (§ 152-52), and/or chronic exposure (§ 152-50) studies are required by 40 CFR 158.165 if the potential for oncogenic, mutagenic, or other adverse chronic effects are indicated, based on:

(i) Results of this study (§ 152-42); and

(ii) The extent of expected human exposure based on residue analysis data developed in accordance with section series 153.

(2) If the potential for oncogenic, mutagenic, or other adverse chronic effects is not indicated, then no further testing is necessary.

§ 152-43 Acute intraperitoneal or intracerebral toxicity/infectivity tests with bacteria, fungi, and protozoa: Tier II.

(a) When required. Data from acute intraperitoneal (IP) or intracerebral (IC) infectivity tests are required by 40 CFR § 158.165 to support the registration of each end-use product for which, in Tier I acute oral infectivity testing (§ 152-30), or dermal toxicity/infectivity testing (§ 152-31), or intraperitoneal and intracerebral injection testing (§ 152-33), the test microorganism (bacteria, fungi, or protozoa) survived for more than 2 weeks, caused toxic effects, or caused a severe illness response in an experimental animal as evidenced by irreversible gross pathology, severe weight loss, toxemia, or death. These data are also required to support the registration of each manufacturing-use product which may legally be used to formulate such an end-use product. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Test standards. The acute IP or IC infectivity test should be conducted in accordance with the standards set forth in § 152-33, with the following exceptions:

(1) Substances to be tested. (i) The technical grade of the active ingredient shall be tested.

(2) Species. Two species other than those used in the comparable Tier I test should be tested. The preferable test species are the rat, guinea pig, rabbit, and beagle dog.

(3) Number of animals and selection of dose levels. Test animals, both normal and immunodepressed, should be injected using at least three graded doses of microorganisms, in sufficient numbers to permit sacrifice at approximately two-week intervals throughout the entire test period. At least five animals per sex, species, dose, and state of immunodepression per sacrifice should be used.

(4) Control animals. A control group, one-half immunodepressed, should be injected (IP or IC as appropriate) with the test vehicle containing the killed (autoclaved) test microorganism. Control animals should be sacrificed concurrently with the treated animal groups. At least five animals per sex, species, and state of immunodepression should be used per sacrifice.

(5) Conduct of test. (i) Application. Approximately 0.1 ml of test solution per graded dose of test microorganism should be injected in accordance with standard procedures and sites for IP and IC tests.

(ii) Duration of test. Surviving animals should be observed for at least four months in tests of bacterial agents and six months in tests of fungal agents, or until the microorganisms are no longer detectable at the injection site or at remote sites.

(c) Data and reporting and evaluation. In addition to the applicable information required by § 80-4 of Subdivision F and § 152-33 of this subdivision, the test report should include the following information:

(1) The LD50 for each sex for each test substance for injected animals calculated at the end of the observation period (with method of calculation specified) expressed in numbers of viable microorganisms per kg body weight and mg product per kg body weight;

(2) The 95 percent confidence interval for the LD50; and

(3) The dose response curve and slope.

(d) Tier progression.

(1) If evidence of microbial agent replication, persistence, or death of test animals is observed, then the Tier III chronic feeding study (§ 152-50) is required by 40 CFR § 158.165.

(2) Data on oncogenicity and/or mutagenicity tests (§§ 152-51 and -52, respectively) are required by 40 CFR § 158.165 if the potential for mutagenic or oncogenic effects are indicated based on:

(i) Results of this test (§ 152-43); and

(ii) The extent of expected human exposure based on residue analysis data developed in accordance with section series 153.

(e) References. Refer to § 152-33(e).

§ 152-44 Primary dermal irritation study with microbial agents: Tier II.

(a) When required. Data from a primary dermal irritation study (Tier II) are required by 40 CFR § 158.165 to support the registration of each end-use product for which marked edema or broad erythema was observed in the Tier I dermal irritation study (§ 152-34).

(b) Test standards. The test standards set forth in § 152-34 apply, except that the use-dilution of the end-use product should be tested.

(c) Data reporting and evaluation. The provisions of § 152-34 apply.

(d) Tier progression. No additional tests are necessasry.

§ 152-45 Primary eye irritation study with microbial agents: Tier II.

(a) When required. Data on primary eye irritation (Tier II) are required by 40 CFR § 158.165 to support the registration of each end-use product for which severe ocular lesions are observed in the Tier I primary eye irritation study (§ 152-35 of this subdivision).

(b) Test standards. The test standards set forth in § 152-35 apply except that the use dilution of the end-use formulated product should be tested.

(c) Data reporting and evaluation. The provisions of § 152-35 apply.

(d) Tier progression. No additional testing is required.

§ 152-46 Effects of microbial pest control agents on the cellular immune response system: Tier II.

(a) When required. Data on cellular immune response (Tier II) are required to support the registration of each end-use formulated product for which results of the Tier I cellular immune response test (§ 152-38 of this subdivision) indicate abnormalities in any of the studies identified in paragraph (a) of § 152-38 and to support the registration of each manufacturing-use product which may legally be used to formulate such an end-use product. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Test standards. [Reserved]

(c) Reporting and evaluation of data. [Reserved]

(d) Tier progression. If results of the cellular immune response test indicate a potential hazard to humans or domestic animals because of the survival or multiplication of the microorganism, then appropriate Tier III tests are required by 40

CFR 158.165. Specific data requirements in Tier III will be determined based on the results of this test (§ 152-46) and consultation with the Agency. Data requirements could include oncogenicity (§ 152-51), chronic feeding (§ 152-50), and/or mutagenicity studies (§§ 152-52).

(e) References.

(1) Refer to § 152-18(f).

(2) The following provides useful information for developing an acceptable protocol for tests to assess antibody-forming activity and cellular immune responses:

> (i) Antibody-forming activity. At least two groups of five mice each are treated with the high level of test substance used in Tier I (§ 152-18). At two time intervals after exposure (15 and 30 days), test and control animals are immunized with a standardized dose of an antigen (sheep erythrocytes; 4 X 10^8 washed red blood cells). Four days later, the animals are sacrificed and the spleen is obtained. The numbers of splenic antibody plaque-forming cells and erythrocytes are determined by a localized hemolysis-in-gel assay using the standard Jerne plaque assay to determine whether there are any untoward affects on anti-body-producing B cell competence.
>
> The plaque assay is described below as an example for both *in vivo* and *in vitro* tests. It is not suitable merely to test the serum antibody levels of the mice, since such testing may be too insensitive to detect changes in numbers of individual antibody producing cells in animals already exposed to detrimental levels of pesticides.
>
> (ii) Cellular immune responses. Groups of pesticide-treated mice should be injected with the technical preparation of the control agent in the appropriate manner to determine whether there has been an effect on cell-mediated immune responses. At least one assay for cell-mediated immunity, either with whole animals or with cells in culture, should be performed for animals treated with the maximum dose of the microbial pest control agent which elicited effects in Tier I testing (§ 152-38).

The test should be performed with groups of at least five animals or more at 15 and 30 days after exposure. Typical in vivo assays would be based on a determination of allogenic skin graft rejection and/or resistance to highly allogenic tumor cells.

(A) In vivo test. Groups of ten mice each are given a full thickness allogenic skin graft, and mean survival time is determined and compared with the survival time of allogenic skin grafts on untreated animals. Injection of a tumor cell line could be performed using mastacytoma tumor cells in which the LD50 is readily established for control animals. It will then be possible to determine whether the pesticidetreated animals show a significant enhanced susceptibility to the same numbers of mastacytoma cells.

(B) In vitro test. Spleen cells from animals sensitized with the normal allogenic skin graft or given tumor cells as described in paragraph (e)(ii)(A) are used for quantitative cell-mediated immune responses in which chromium-labeled target cells are exposed to splenocytes from sensitized animals in vitro in order to ascertain responsiveness of killer T lymphocytes in the treated animal spleen. Lymphoid cells (10^6) obtained from sensitized animals - either control animals or animals treated 15 and 30 days previously with a maximum dose of pesticide - are tested in a chromium release assay with labeled target cells in cultures. In addition, lymphoid cells from treated animals are to be tested for their ability to generate migration inhibitory factor in vitro. For these tests, splenocytes (10^6) are placed in microcapillary tubes, with or without a specific antigen (such as the purified protein derivative or mycobacteria if the animals were sensitized with the BCG strain of mycobacterium or extracts of the allografts). The ability of the mononuclear spleen cell suspensions to migrate from the chamber in the presence of antigen would be used as a correlate of cellular immunity. As an alternative to one of the above procedures, a blastogenic test could be performed. Spleen cells (10^6) are cultured in microwell chambers with and without a stimulator such as phytohemaglutinin, concanavalin

A, or lipopolysaccharide. The ability of the spleen cells to respond to these mitogens in vitro by taking up tritiated thymidine corresponds to an assessment of cellular immunity.

§ 152-47 Teratogenicity test with viral agents: Tier II.

(a) When required. Data on teratogenicity studies are required by 40 CFR § 158.165 to support the registration of each end-use product for which the Tier I tests on viral agents (§ 152-30 through -33 and -39) show replication of the virus in mammalian hosts and significant damage to mammalian cells and to support the rgistration of each manufacturing-use product which may legally be used to formulate such an end-use product. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Test standards. The applicable general test standards and applicable specific test standards set forth in § 80-3 and § 83-3(e) through (g), respectively, of Subdivision F, apply, with the following exceptions:

(1) Substance to be tested. Testing shall be performed with the technical grade of the active ingredient (virus).

(c) Data reporting and evaluation. The applicable provisions of § 83-3 of Subdivision F apply.

(d) Tier progression. If evidence of teratogenic effects due to treatment with the viruses is demonstrated, then additional testing in Tier III may be required by 40 CFR § 158.165 following consultation with the Agency.

§ 152-48 Bacterial and fungal virulence enhancement: Tier II.

(a) When required. Data from a bacterial or fungal virulence enhancement study are required by 40 CFR § 158.165 to support the registration of each end-use product containing bacteria or fungi as the active ingredient when Tier I infectivity tests (§ 152-30 or -33) indicate prolonged survival (including presence of viable microbial agents in test animal excreta) and/or multiplication (infectivity) of the bacterial or fungal agent, respectively. These data are also required to support the registration of each manufacturing-use product which may legally be used to formulate such an end-use product. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this Subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Test standards. In addition to the applicable general standards set forth in § 150-3 of this subdivision and § 80-3 of Subdivision F, bacterial or fungal virulence enhancement studies should meet the following standards:

(1) Substance to be tested. The technical grade of the active ingredient shall be tested.

(2) Age, sex and species. Testing should be performed on the adult male and female laboratory mouse or hamster.

(3) Number of animals. At least 10 animals per dose level, at least three dose levels to conduct each of three LD50 assays, and at least 20 animals per each of five separate serial passage experiments should be provided.

(4) Conduct of test. An initial intraperitoneal LD50 study (expressed as numbers of microorganisms per kg body weight and mg product per kg body weight) to determine virulence should be performed. The test bacteria or fungi shall be serially passed through mice or hamsters at least five times, in order to evaluate potential for virulence enhancement. These steps should be followed by a final IP LD50 to detect possible virulence enhancement, expressed as numbers of microorganisms, per kg body weight and mg product per kg body weight, following final passage.

(i) Initial intraperitoneal LD50. An IP LD50 should be performed using at least three different dose levels of the test bacteria or fungi suspended in physiological saline administered to groups of at least 10 animals each. Animals should be observed for mortality during a two-week period.

(ii) Serial passage. Two groups of at least 10 each should be injected intraperitoneally separately with suspensions of the test bacteria or fungi in physiological saline, and an adjuvant such as Freund's adjuvant, or gastric mucin. Moribund animals and animals surviving a two-week observation period should be sacrificed. Attempts to recover the test bacteria or fungi from animals dying during testing or at terminal sacrifice should be made by culturing samples of blood, liver, spleen, and kidneys on suitable media. Bacteria or fungi thus recovered and identified from tissues should be further cultured or inoculated directly following suspension in saline or adjuvant into similar groups of untreated animals, thus completing one serial passage. This process should be repeated through at least five serial passages.

(iii) Final intraperitoneal LD50. IP LD50's conducted in the same manner as the initial IP LD50 should be performed with test bacteria or fungi recovered from the fifth passage:

(A) An IP LD50 performed with serially-passed bacteria or fungi suspended in saline; and

(B) An IP LD50 performed with serially-passed bacteria or fungi suspended in an adjuvant.

(c) Data reporting and evaluation. In addition to the information in § 80-4 and paragraphs (b)(4)(i), (ii) and (iii) of this section, the test report should include the following information:

(1) Tabulation of response data by sex and dose level (i.e., number of animals showing signs of toxicity per number of animals exposed);

(2) Time of death after dosing;

(3) IP LD50's performed prior to serial passage, and IP LD50's performed following serial passage in mice (specify method of LD50 calculation) expressed as numbers of bacteria or fungi per kg body weight:

(i) Using inoculum suspended in physiological saline; and

(ii) Using inoculum suspended in an adjuvant.

(4) 95 percent confidence interval for the LD50 expressed as numbers of organisms;

(5) Dose response curve and slope;

(6) For each animal subjected to necropsy, the kinds of tissues from which the test bacteria or fungi could be recovered and identified per tissues cultured should be reported; and

(7) Compare number of organisms for initial and final IP LD50 values; reduced numbers of microorganisms per kg body weight for final IP LD50 values would indicate increased virulence.

(d) Tier progression. (1) If evidence of virulence enhancement is observed with bacterial or fungal agents then a chronic feeding study (§ 152-50) is required as specified by 40 CFR 158.165.

(2) If no evidence of virulence enhancement is observed, no further testing is necessary.

§ 152-49 Mammalian mutagenicity testing with microbial pest control agents: Tier II.

(a) When required. Data from short-term mammalian mutagenicity tests, as specified in § 152-19, are required by 40 CFR 158.165 to support the registration of each end-use product that meets any of the criteria presented in (1)-(3), below and to support the registration of each manufacturing-use product which may legally be used to formulate such an end-use product. See 40 § CFR 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(1) Acute infectivity tests are positive in Tier I studies (as specified in §§ 152-30 through -33 of this subdivision);

(2) Adverse cellular effects are observed in cellular immune response studies (as specified in Tier I § 152-38); or,

(3) Positive results are obtained in tissue culture tests with viral agents (as specified in § 152-39).

(b) Test standards. Tests should be performed according to both the general and specific standards set forth in § 152-19 of this subdivision, with any necessary modifications due only to the nature of the test substance.

(c) Test substance. The technical form of the active ingredient shall be tested. In addition, the purest infective form (PIF) shall be tested for viral agents.

(d) Reporting. The information to be reported is the same as that specified in § 152-19 of this subdivision with any necessary modifications due to the nature of the test substance.

(e) Tier progression. Positive results for mutagenicity in any mammalian cell or organism test will require additional mutagenicity testing in Tier III (§ 152-52) and/or oncogenicity testing (§ 152-51) as specified in 40 CFR § 158.165. The specific test required in Tier III shall be determined after consultation with the Agency.

(f) References. Refer to § 152-19.

Group B-3: Tier III Testing.

§ 152-50 Chronic feeding study: Tier III.

(a) When required. Data on a chronic feeding study are required by 40 CFR § 158.165 to support the registration of each end-use product for which the potential for chronic adverse effects (e.g., replication or persistence of viral or subviral constituents, protozoans, fungi, or bacteria) are demonstrated by any of the following Tier II tests: §§ 152-40 through -43, -46, and -49. These data are also required to support the registration of each manufacturing-use product which may legally be used to formulate such an end-use product. Consultation with the Agency is advised before performing these studies. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Combined testing. A chronic feeding study may be combined with an oncogenic evaluation pursuant to § 152-51, provided that standards for both types of testing are met.

(c) Test standards. The applicable general and specific standards set forth in §§ 80-3 and 83(d) and (e), respectively, of Subdivision F apply, with any necessary modifications due to the nature of the test substance.

(d) Data reporting and evaluation. The provisions set forth in § 83-1 of Subdivision F shall apply, with any necessary modifications due to the nature of the test substance.

§ 152-51 Oncogenicity studies: Tier III.

(a) When required. Data from oncogenicity testing may be required by 40 CFR § 158.165 to support the registration of each end-use product for which the potential for oncogenic effects is indicated (e.g., adverse cellular effects due to presence, replication, or persistence of viral or subviral constituents, or bacteria, fungi or protozoans; or mutagenic effects) by any of the following Tier II tests: §§ 152-40 through -43, -46, and -49. These data are also required to support the registration of each manufacturing-use product which may legally be used to formulate such an end-use product. Consultation with the Agency is advised before performing these studies. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Combined testing. A chronic feeding study may be combined with an oncogenic evaluation pursuant to § 152-50, provided that standards for both types of testing are met.

(c) Test standards. The applicable general and specific standards set forth in §§ 80-3 and 83(d) and (e), respectively, of Subdivision F apply, with any necessary modifications due to the nature of the test substance.

(d) Data reporting and evaluation. The provisions set forth in § 83-2 of Subdivision F shall apply, with any necessary modifications due to the nature of the test substance.

§ 152-52 Mutagenicity testing: Tier III.

(a) When required. Data from whole animal mutagenicity testing may be required by 40 CFR § 158.165 to support the registration of each end-use product for which the potential for mutagenic effects is indicated (e.g., adverse cellular effects due to presence, replication, or persistence of viral or subviral constituents, bacteria, fungi or protozoa) by any of the following Tier II tests: §§ 152-40 through -43, and -46 or -49. These data are also required to support the registration of each manufacturing-use product which may legally be used to formulate such an end-use product. Consultation with the Agency is advised before performing this testing. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Test standards. The applicable general and specific standards set forth in §§ 80-3 and 84-1 through -4 of Subdivision F apply, with any necessary modifications due to the nature of the test substance.

(c) Data reporting and evaluation. The provisions set forth in §§ 84-1 through -4 of Subdivision F apply, with any necessary modifications due to the nature of the test substance.

(d) References. Refer to § 84-5 of Subdivision F.

§ 152-53 Teratogenicity studies: Tier III.

(a) When required. Data on teratogenicity studies are required by 40 CFR § 158.165 to support the registration of each end-use product for which the potential for teratogenic effects is expected based on the presence or persistence of fungi, bacteria, viruses, or protozoa in mammalian species as a result of testing performed in Tier II (e.g., §§ 152-40 through -43, -46, -48, and -49). These data are also required to support the registration of each manufacturing-use product which may legally be used to formulate such an end-use product. Consultation with the Agency is advised before performing these studies. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Test standards. The applicable general and specific standards set forth in §§ 80-3 and 83-3(e) through (g), respectively, of Subdivision F apply, with any necessary modifications due to the nature of the test substance.

(c) Data reporting and evaluation. The provisions set forth in § 83-3 of Subdivision F apply, with any necessary modifications due to the nature of the test substance.

Series 153: RESIDUE ANALYSIS GUIDELINES FOR BIORATIONAL PESTICIDES

§ 153-1 Overview.

(a) Requirements. A petition for a tolerance or for an exemption from the requirement of a tolerance must be submitted as specified in 40 CFR § 158.165 in connection with each application for registration of a biorational pesticide product where usage may result in residues in or on food for humans or feed for domestic animals used for human food. This petition must contain data satisfying the requirements of 40 CFR § 158.165 which are detailed in this section series (153) unless specifically exempted from the requirements.

(b) Purpose. Residue chemistry data are designed to provide the information necessary to determine the site, nature, and magnitude of residues in or on food or feed. This information includes plant metabolism data, residue data, analytical methodology, and, when indicated, animal metabolism data and animal feeding studies to determine the carryover of residues into meat, milk, poultry, and eggs.

(c) Authority. Pesticides intended for use on food or feed crops, or where usage may reasonably be expected to result (directly or indirectly) in residues in food or feed, will not be registered unless a tolerance, or an exemption from the requirement of a tolerance, has been established by the Agency, as provided for under Sections 406, 408, or 409 of the Federal Food, Drug, and Cosmetic Act ("FFDCA" 21 U.S.C. 346, 346a and 348). The procedural regulations for filing petitions for a tolerance or an exemption are included in 40 CFR § 180.7.

(d) Location of guidelines. § 153-3 lists the guidelines for biochemical pest control agents and § 153-4 refers to guidelines for microbial pest control agents.

§ 153-2 [Reserved]

§ 153-3 Residue data guidelines for biochemical pest control agents.

(a) When required. (1) A petition for a tolerance or for an exemption from the requirement of a tolerance is required by 40 CFR § 158.165 in connection with each application for the registration of each end-use product and manufacturing-use product composed of or containing a biochemical when the following conditions are met:

(i) The product is intended for use on food or feed crops or its use is expected to result in residues in or on food or feed; and

(ii) The rate of application exceeds 0.7 ounces (20 grams) of the biochemical (active ingredient) per acre per application.

(2) Residue data requirements will be determined on a case-by-case basis for biochemicals applied directly to food or feed and for biochemicals with application rates not expressable in ounces per acre per application.

(3) Residue data will not be required and an exemption from the requirement of a tolerance will be recommended for products intended for use on food or feed crops or for uses expected to result in residues in or on food or feed, when the following conditions are met:

(i) Toxicology data developed from Tier I testing in accordance with §§ 152-10 through -18 of this subdivision indicate that testing at Tier II is not required; and

(ii) The rate of application is equal to or less than 20 grams of biochemical (active ingredient) per acre per application.

(b) Procedures, standards, and reporting. (1) General. In addition to the applicable general provisions of §§ 150-3 and -4, the information provided in Subdivision O (Series 170) should be used as general guidelines for test procedures, test standards, and the reporting of data.

(2) Specific aspects. (i) The full range of residue chemistry data detailed in Subdivision O (Series 170) apply to products for which:

(A) Toxicity testing proceeds to Tier II or III, as described in §§ 152-19 through -29 of this subdivision, or

(B) The application rate exceeds 0.7 ounces (20 grams) active ingredient per acre per application.

(ii) A petition for a tolerance or an exemption from the requirements of a tolerance shall be required in connection with each application for registration for each biochemical that meets either of the criteria set forth in paragraph (b)(2)(i)(A) or (B) of this section.

§ 153-4 Residue data requirements for microbial pest control agents.

(a) When required. Residue data are required by 40 CFR § 158.165 to be included in a petition for a tolerance or for an exemption from the requirement of a tolerance, in connection with each application for registration of a manufacturing-use product or end-use product composed of or containing a microbial agent, when the following conditions are met:

(1) When the product is intended for use on food or feed crops, or

(2) When use of the product is expected to result in residues in or on food or feed; and

(3) When results of Tier I toxicology studies conducted in accordance with §§ 152-30 through -39 of this subdivision indicate that Tier II toxicology studies described in §§ 152-40 through -48 are required.

(4) Residue data will not be required and an exemption from the requirement of a tolerance will be recommended for products intended for use on food or feed crops or for uses expected to result in residues in or on food or feed, when the toxicology data developed from Tier I testing, in accordance with §§ 152-30 through - 39 of this subdivision, indicate that testing at Tier II is not required.

(b) Procedures, standards, and reporting. In addition to the provisions set forth in §§ 150-3 and -4 that are applicable, the following guidance is provided for conducting, developing, and reporting the residue data that the Agency requires to support a petition for a tolerance or for an exemption from the requirement of a tolerance:

(1) Subdivision O (Series 170) contains applicable information. For the qualification of viable bacteria or fungi (or their spore forms), artificial media may be employed following washing, homogenization, centrifugation, or other appropriate treatments to collect and/or concentrate the organism. With viruses and protozoa, specific hosts may be required for quantitative measurements of viable residues. These recovery techniques may be supplemented or even replaced by appropriate proven techniques involving fluorescent antibodies or electron microscopy.

(2) Discussion with appropriate Agency scientists may be helpful before steps are taken to develop residue data of the nature outlined in this section.

Series 154: NONTARGET ORGANISM HAZARD GUIDELINES FOR BIORATIONAL PESTICIDES

§ 154-1 General information.

(a) Scope of guidelines for nontarget organisms. Section series 154 outlines the Agency guidelines for the submission of data and information relating to pesticidal effects on terrestrial wildlife, aquatic animals, plants, and beneficial insects in support of applications for registration of naturally occurring or synthetic biochemical pest control agents and microbial pest control agents. In many instances these guidelines refer to other subdivisions pertaining to conventional pesticide products.

(b) Approach. The tests to evaluate pesticidal effects on terrestrial animals, aquatic animals, plants, and beneficial insects are arranged in a hierarchical or tier system, beginning with acute testing at Tier I. Tier II testing involves Environmental Fate testing for biochemical agents and Environmental Expression testing for microbial agents (section series 155) to estimate environmental concentrations of pesticides after application. Tier III consists of further acute, subacute, and chronic laboratory testing on nontarget organisms, and Tier IV consists of applied field tests encompassing both nontarget organisms and environmental fate or expression. The results of each tier of tests must be evaluated to determine whether further testing is necessary. It is expected that the extent of testing will diminish with each subsequent tier. (Figures 1 through 5 illustrate the tier testing systems for biochemicals for terrestrial wildlife, aquatic animals, plants, and terrestrial and aquatic insects, respectively. Figures 6 through 10 illustrate the tier testing systems for microbial pest control agents for terrestrial wildlife, aquatic animals, plants, and terrestrial and aquatic insects, respectively.)

(c) Organization. The guidelines are divided into two broad categories - those for biochemicals and those for microbial pest control agents. The protocols to develop the data are organized in a tier arrangement within each of these categories and each tier contains testing protocols pertaining to the major groups of nontarget organisms (terrestrial wildlife, aquatic animals, plants and insects). Table 6 illustrates this organization.

§§ 154-2 through -5 [Reserved].

Figure 1--TERRESTRIAL WILDLIFE TIER TESTING SCHEME FOR BIOCHEMICAL PESTICIDES

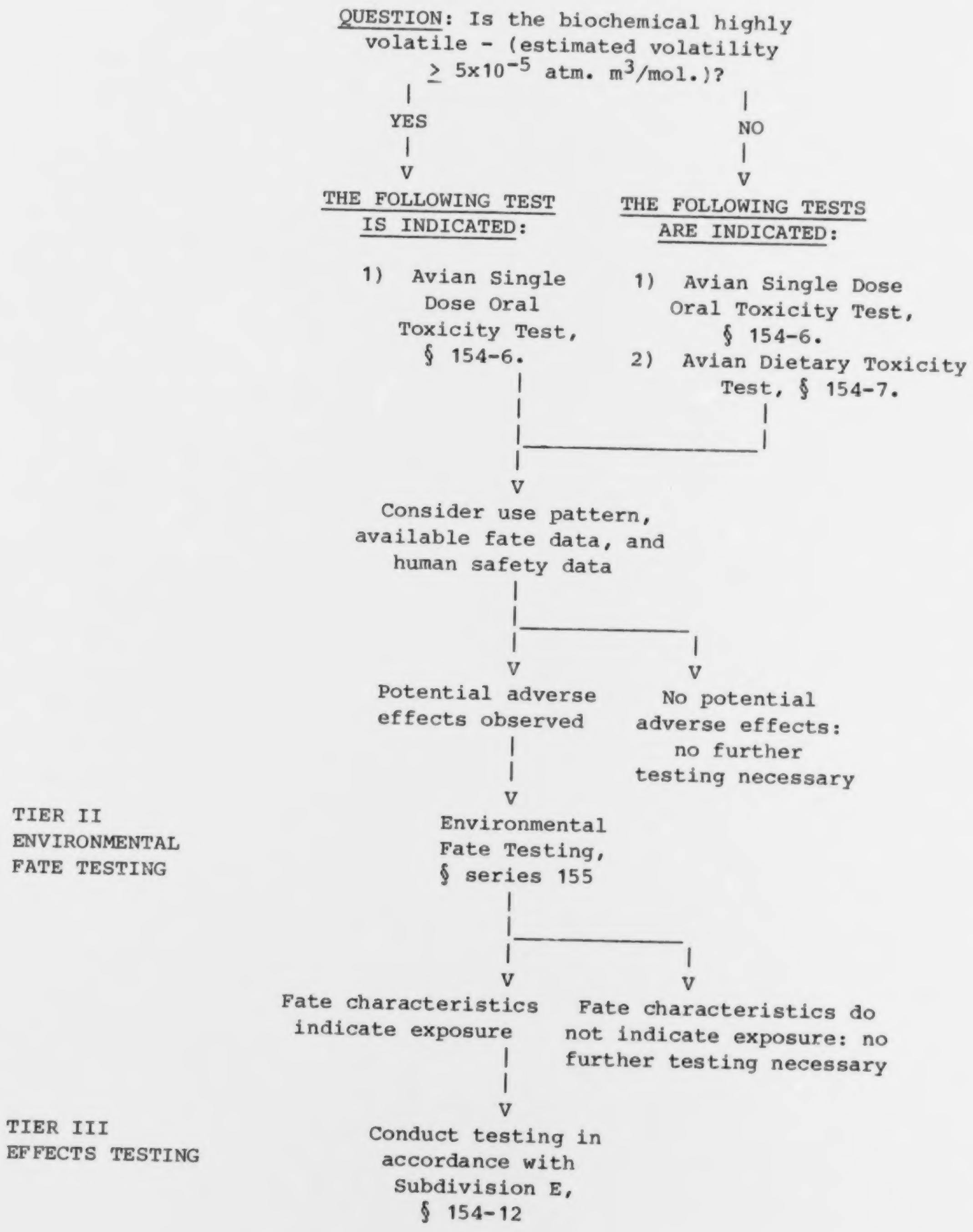
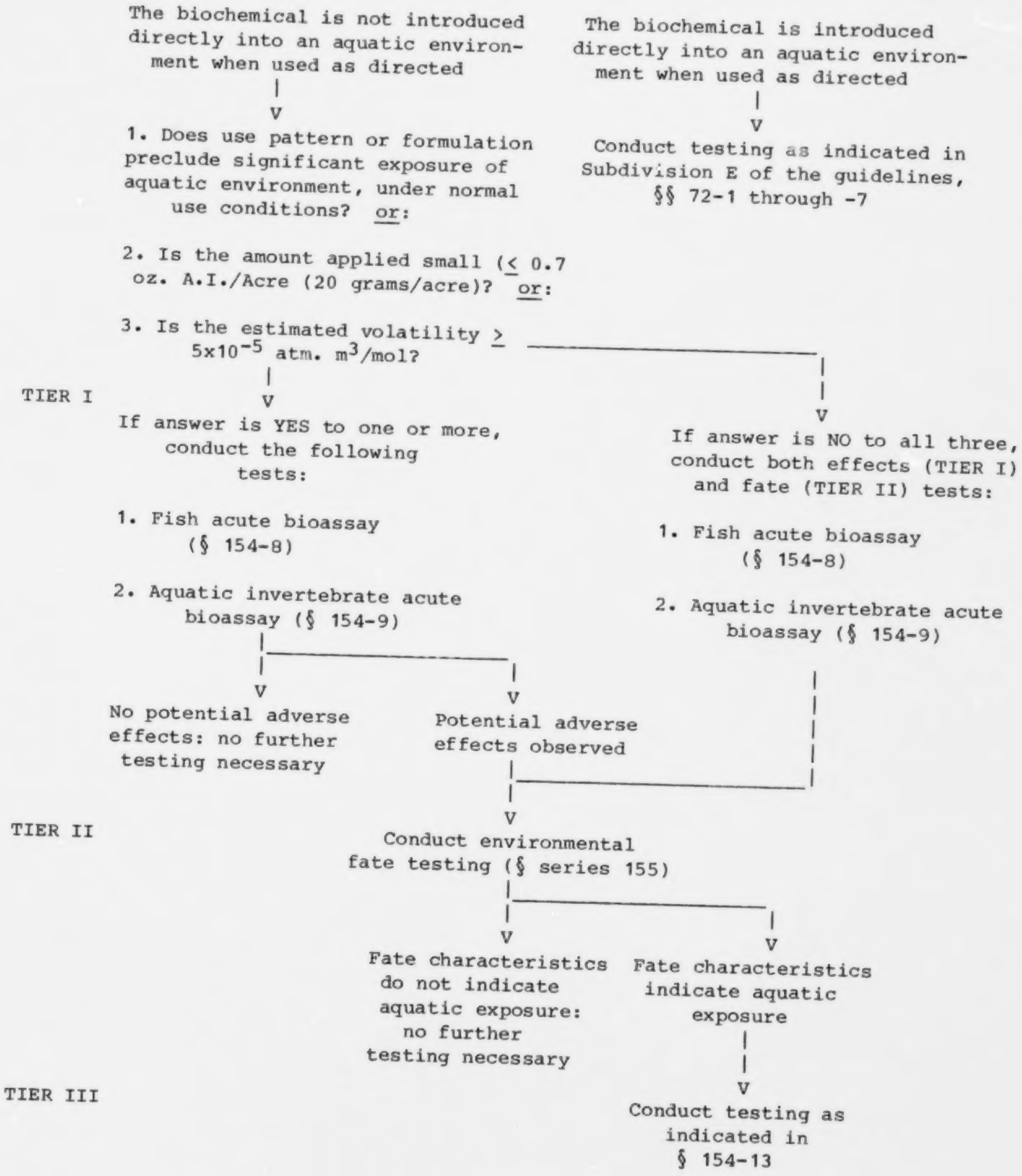

QUESTION: Is the biochemical highly volatile - (estimated volatility $\geq 5\times10^{-5}$ atm. m^3/mol.)?
YES
NO
THE FOLLOWING TEST IS INDICATED:
1) Avian Single Dose Oral Toxicity Test, § 154-6.
THE FOLLOWING TESTS ARE INDICATED:
1) Avian Single Dose Oral Toxicity Test, § 154-6.
2) Avian Dietary Toxicity Test, § 154-7.
Consider use pattern, available fate data, and human safety data
Potential adverse effects observed
No potential adverse effects: no further testing necessary
TIER II ENVIRONMENTAL FATE TESTING
Environmental Fate Testing, § series 155
Fate characteristics indicate exposure
Fate characteristics do not indicate exposure: no further testing necessary
TIER III EFFECTS TESTING
Conduct testing in accordance with Subdivision E, § 154-12

Figure 2--AQUATIC ANIMAL TIER TESTING SCHEME FOR BIOCHEMICAL PESTICIDES



The biochemical is not introduced directly into an aquatic environment when used as directed

1. Does use pattern or formulation preclude significant exposure of aquatic environment, under normal use conditions? or:

2. Is the amount applied small ($\leq$ 0.7 oz. A.I./Acre (20 grams/acre)? or:

3. Is the estimated volatility $\geq$ 5×10^{-5} atm. m^3/mol?

The biochemical is introduced directly into an aquatic environment when used as directed

Conduct testing as indicated in Subdivision E of the guidelines, §§ 72-1 through -7

TIER I

If answer is YES to one or more, conduct the following tests:

1. Fish acute bioassay (§ 154-8)

2. Aquatic invertebrate acute bioassay (§ 154-9)

If answer is NO to all three, conduct both effects (TIER I) and fate (TIER II) tests:

1. Fish acute bioassay (§ 154-8)

2. Aquatic invertebrate acute bioassay (§ 154-9)

No potential adverse effects: no further testing necessary

Potential adverse effects observed

TIER II

Conduct environmental fate testing (§ series 155)

Fate characteristics do not indicate aquatic exposure: no further testing necessary

Fate characteristics indicate aquatic exposure

TIER III

Conduct testing as indicated in § 154-13

Figure 3--NONTARGET PLANT TIER TESTING SCHEME FOR BIOCHEMICAL PEST CONTROL AGENTS.

TIER I. Consider the mode of action and use:

(Tests to be conducted on a case-by-case basis as specified in 40 CFR § 158.165)

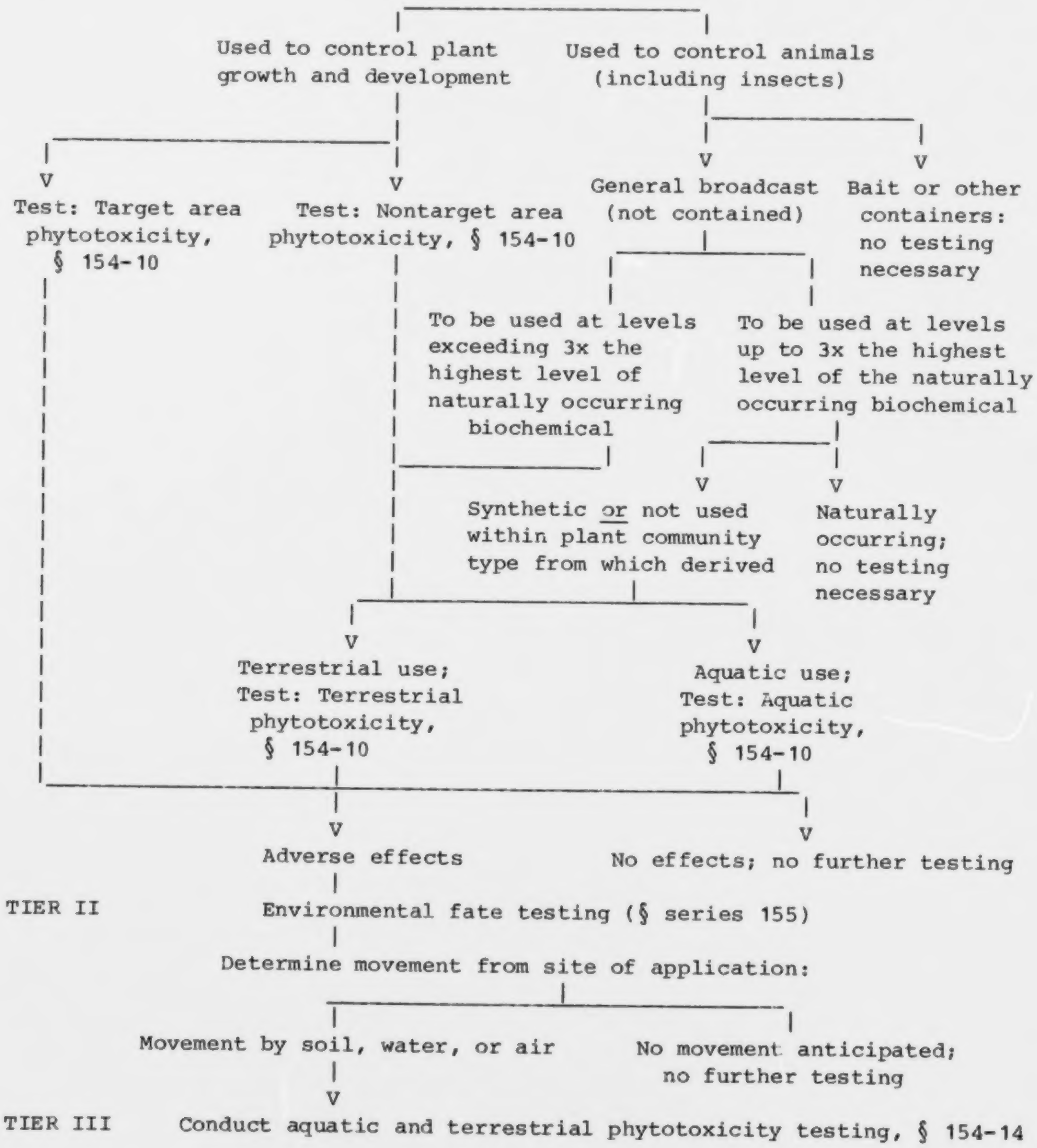

Used to control plant growth and development
Used to control animals (including insects)
Test: Target area phytotoxicity, § 154-10
Test: Nontarget area phytotoxicity, § 154-10
General broadcast (not contained)
Bait or other containers: no testing necessary
To be used at levels exceeding 3x the highest level of naturally occurring biochemical
To be used at levels up to 3x the highest level of the naturally occurring biochemical
Synthetic or not used within plant community type from which derived
Naturally occurring; no testing necessary
Terrestrial use; Test: Terrestrial phytotoxicity, § 154-10
Aquatic use; Test: Aquatic phytotoxicity, § 154-10
Adverse effects
No effects; no further testing
TIER II
Environmental fate testing (§ series 155)
Determine movement from site of application:
Movement by soil, water, or air
No movement anticipated; no further testing
TIER III
Conduct aquatic and terrestrial phytotoxicity testing, § 154-14

Figure 4--NONTARGET INSECT TESTING SCHEME FOR BIOCHEMICAL PESTICIDES - TERRESTRIAL INSECTS

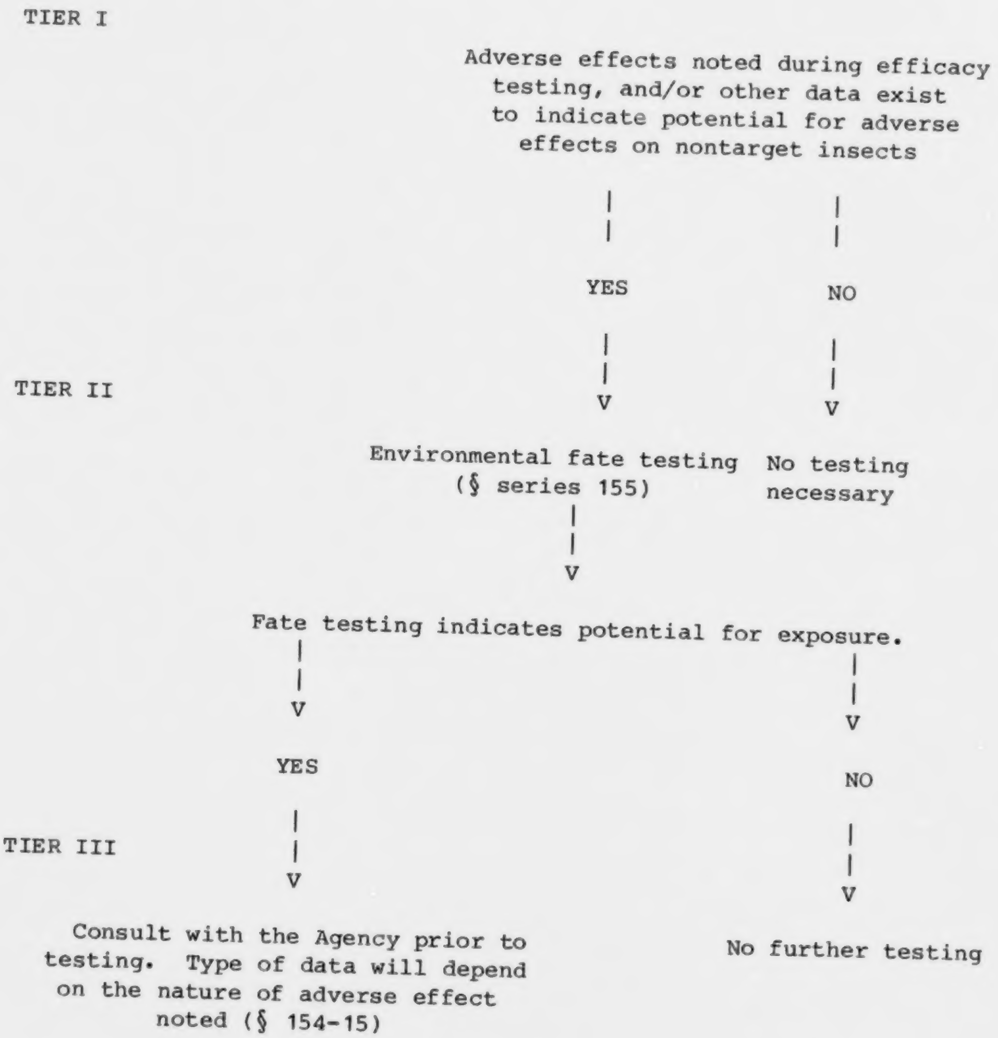
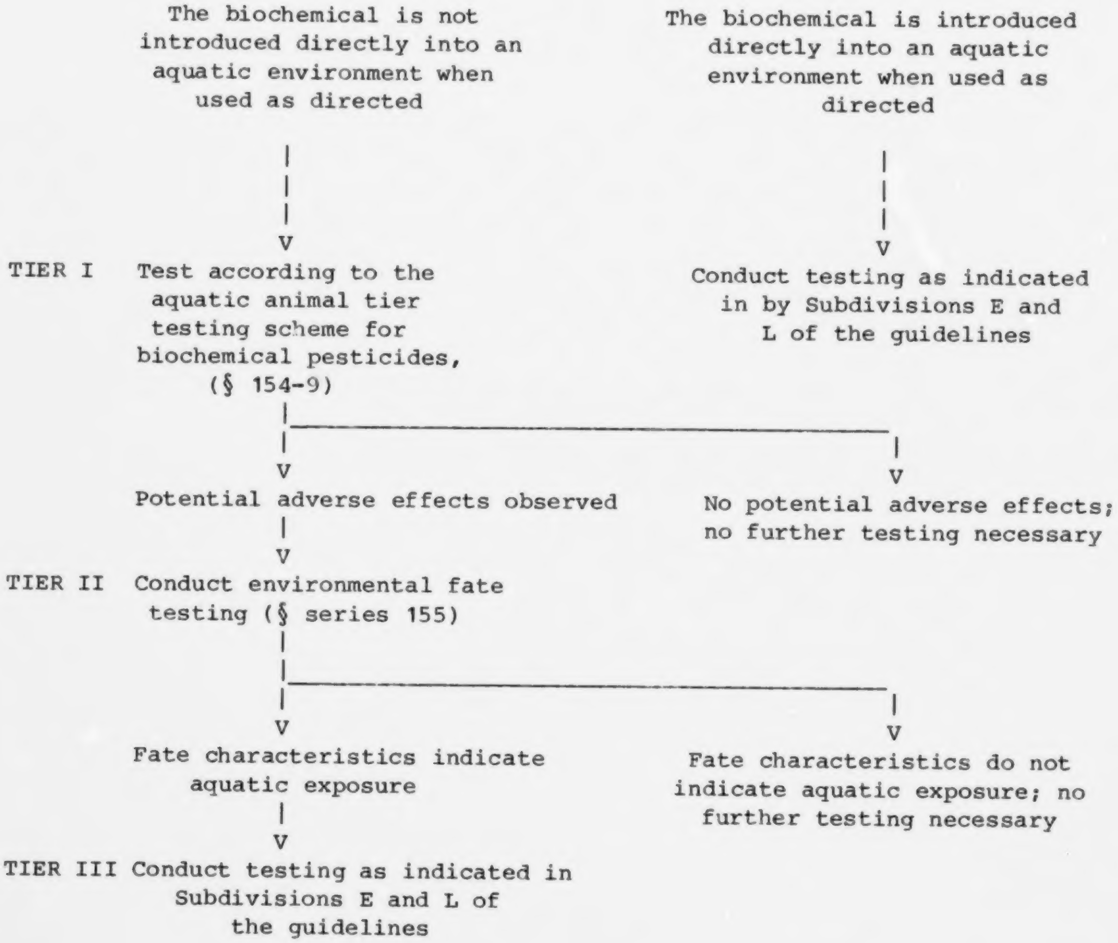

TIER I
Adverse effects noted during efficacy testing, and/or other data exist to indicate potential for adverse effects on nontarget insects
YES
NO
TIER II
Environmental fate testing (§ series 155)
No testing necessary
Fate testing indicates potential for exposure.
YES
NO
TIER III
Consult with the Agency prior to testing. Type of data will depend on the nature of adverse effect noted (§ 154-15)
No further testing

Figure 5--NONTARGET INSECT TESTING SCHEME FOR BIOCHEMICAL PESTICIDES - AQUATIC INSECTS



The biochemical is not introduced directly into an aquatic environment when used as directed

The biochemical is introduced directly into an aquatic environment when used as directed

TIER I Test according to the aquatic animal tier testing scheme for biochemical pesticides, (§ 154-9)

Conduct testing as indicated in by Subdivisions E and L of the guidelines

Potential adverse effects observed

No potential adverse effects; no further testing necessary

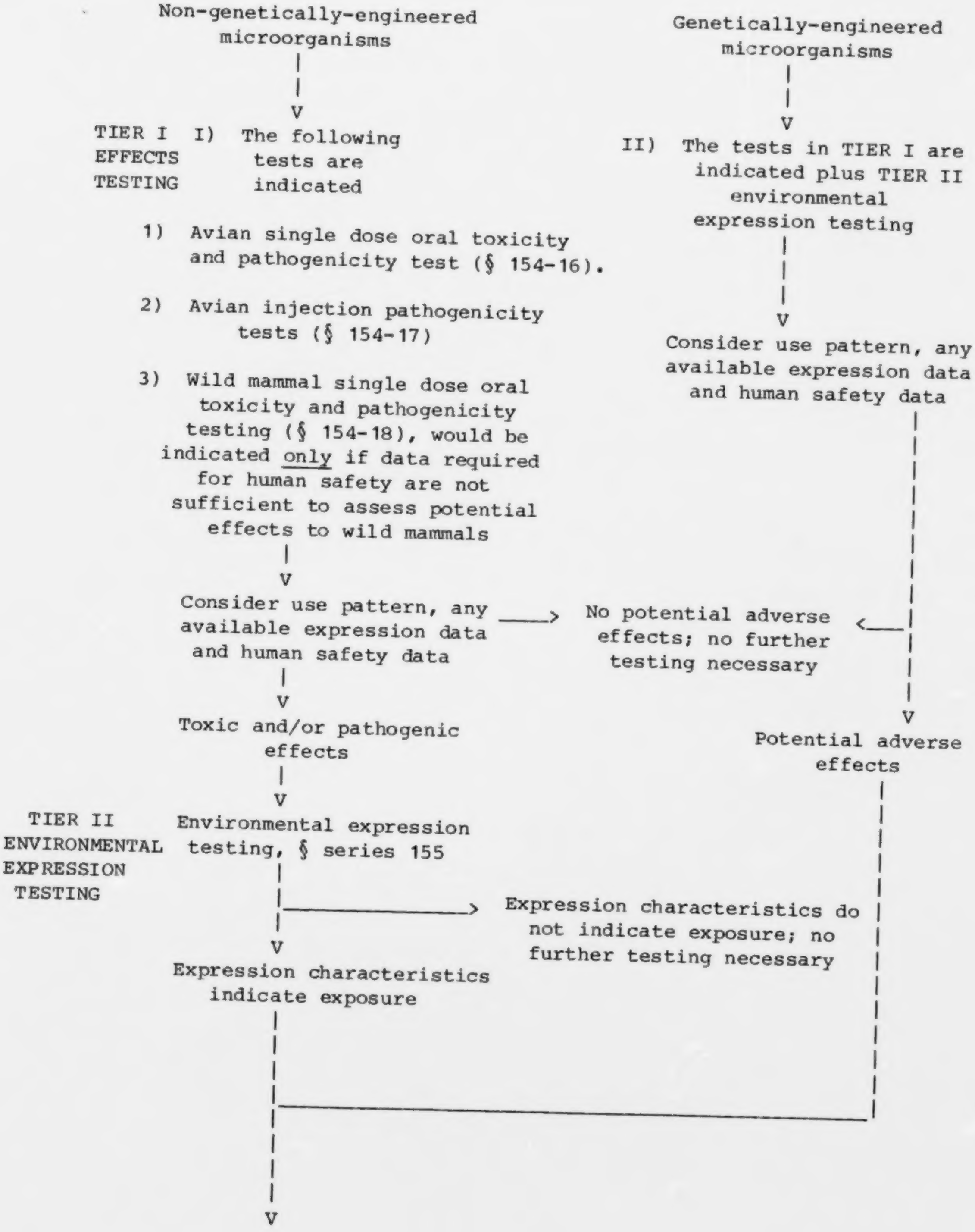
TIER II Conduct environmental fate testing (§ series 155)

Fate characteristics indicate aquatic exposure

Fate characteristics do not indicate aquatic exposure; no further testing necessary

TIER III Conduct testing as indicated in Subdivisions E and L of the guidelines

Figure 6--TERRESTRIAL WILDLIFE TIER TESTING SCHEME FOR MICROBIAL PEST CONTROL AGENTS



Non-genetically-engineered microorganisms

TIER I EFFECTS TESTING

I) The following tests are indicated

1) Avian single dose oral toxicity and pathogenicity test (§ 154-16).

2) Avian injection pathogenicity tests (§ 154-17)

3) Wild mammal single dose oral toxicity and pathogenicity testing (§ 154-18), would be indicated only if data required for human safety are not sufficient to assess potential effects to wild mammals

Consider use pattern, any available expression data and human safety data

Toxic and/or pathogenic effects

TIER II ENVIRONMENTAL EXPRESSION TESTING

Environmental expression testing, § series 155

Expression characteristics indicate exposure

Expression characteristics do not indicate exposure; no further testing necessary

Genetically-engineered microorganisms

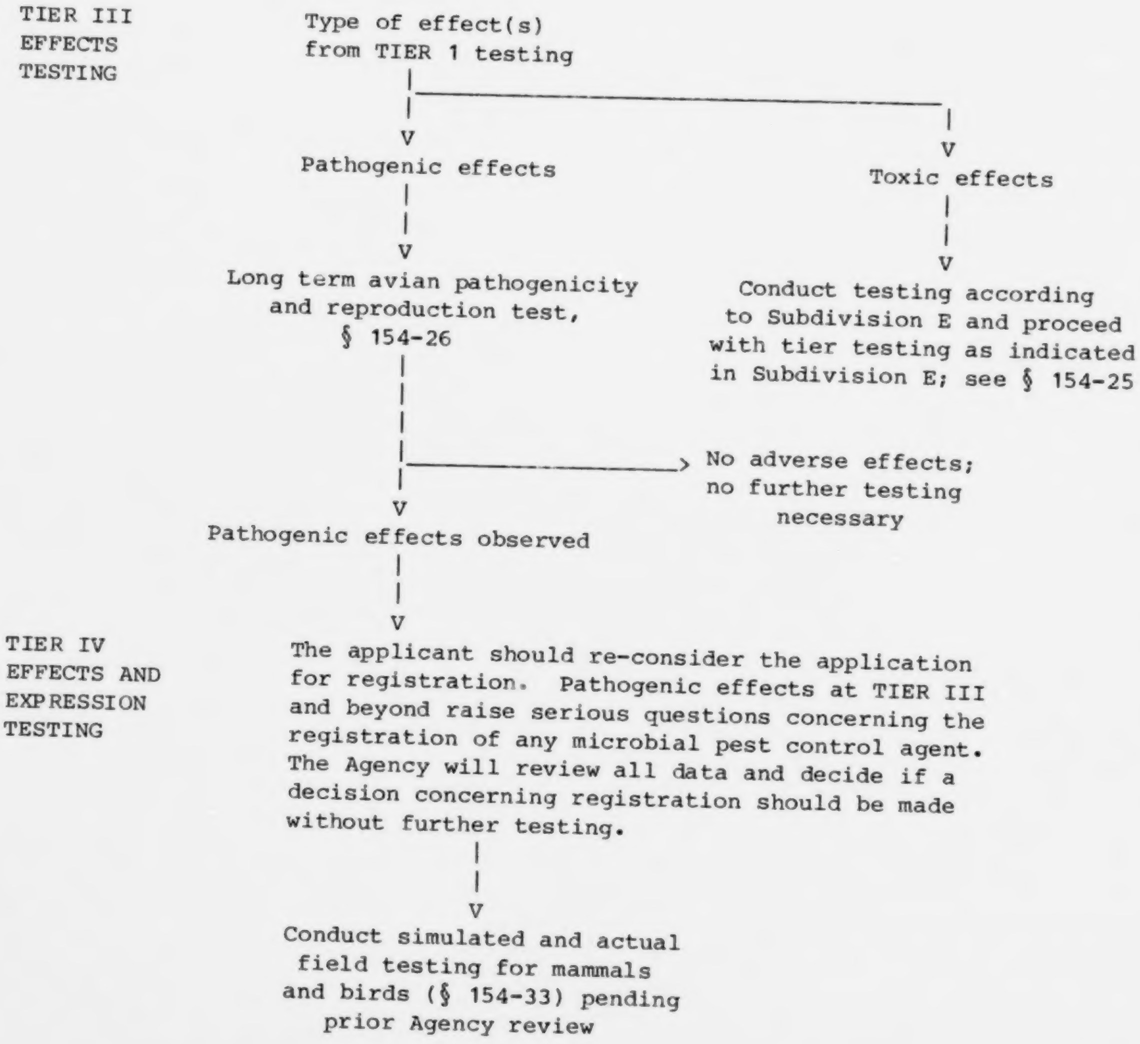
II) The tests in TIER I are indicated plus TIER II environmental expression testing

Consider use pattern, any available expression data and human safety data

No potential adverse effects; no further testing necessary

Potential adverse effects

Figure 6--(continued)



TIER III EFFECTS TESTING

Type of effect(s) from TIER 1 testing

Pathogenic effects

Toxic effects

Long term avian pathogenicity and reproduction test, § 154-26

Conduct testing according to Subdivision E and proceed with tier testing as indicated in Subdivision E; see § 154-25

No adverse effects; no further testing necessary

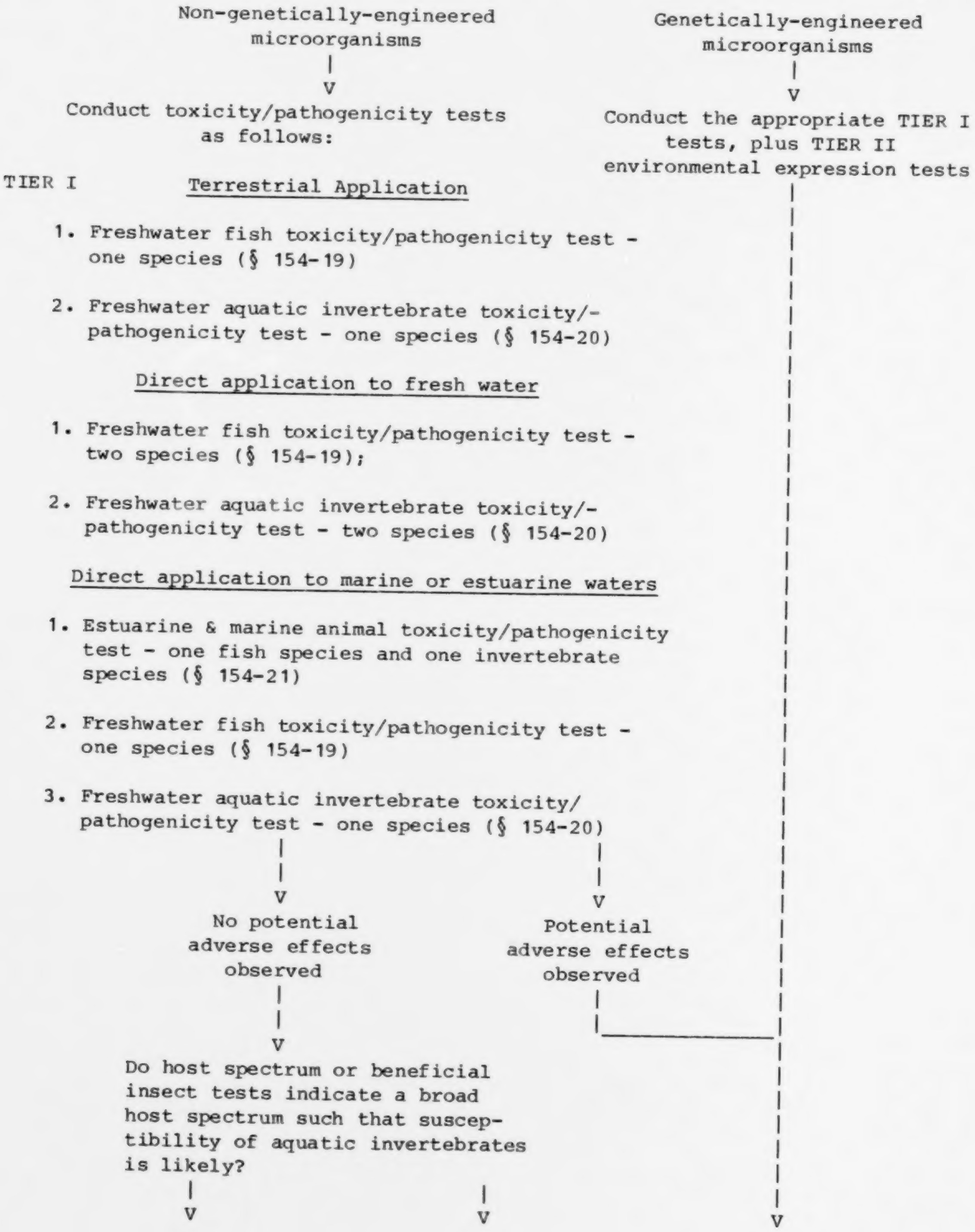
Pathogenic effects observed

TIER IV EFFECTS AND EXPRESSION TESTING

The applicant should re-consider the application for registration. Pathogenic effects at TIER III and beyond raise serious questions concerning the registration of any microbial pest control agent. The Agency will review all data and decide if a decision concerning registration should be made without further testing.

Conduct simulated and actual field testing for mammals and birds (§ 154-33) pending prior Agency review

Figure 7--AQUATIC ANIMAL TIER TESTING SCHEME FOR MICROBIAL PEST CONTROL AGENTS



Non-genetically-engineered microorganisms

Conduct toxicity/pathogenicity tests as follows:

TIER I

Terrestrial Application

1. Freshwater fish toxicity/pathogenicity test - one species (§ 154-19)
2. Freshwater aquatic invertebrate toxicity/-pathogenicity test - one species (§ 154-20)

Direct application to fresh water

1. Freshwater fish toxicity/pathogenicity test - two species (§ 154-19);
2. Freshwater aquatic invertebrate toxicity/-pathogenicity test - two species (§ 154-20)

Direct application to marine or estuarine waters

1. Estuarine & marine animal toxicity/pathogenicity test - one fish species and one invertebrate species (§ 154-21)
2. Freshwater fish toxicity/pathogenicity test - one species (§ 154-19)
3. Freshwater aquatic invertebrate toxicity/ pathogenicity test - one species (§ 154-20)

No potential adverse effects observed

Do host spectrum or beneficial insect tests indicate a broad host spectrum such that susceptibility of aquatic invertebrates is likely?

Potential adverse effects observed

Genetically-engineered microorganisms

Conduct the appropriate TIER I tests, plus TIER II environmental expression tests

Figure 7--(continued)

```
                       |                       |                       |_____
                       V                       V                             |
           If NO, then no further         If YES, either:                    |
           testing is necessary               1.  Test additional aquatic    |
                                ______________    invertebrates (§ 154-20);  |
                               |           |      or:                        |
                               |           |  2.  Conduct TIER II testing    |
                               |           |      (§ series 155)             |
                               V           |                                 |
           Additional species tested,      |->  Additional species tested,   |
           no potential adverse effects;        potential adverse effects    |
           no further testing necessary         observed        |____________|
                                                                             V
TIER II                                                         Conduct environmental
                                        __________________________expression testing
                                        |                          (§ series 155)
                                        |                                |
                                        V                                V
                           No exposure indicated;     _____ Environmental expression
                            no further testing       |         analysis indicates
                                 necessary           |              exposure
                                                     V
                                   ______    Further testing     __________
                                  |         indicated TIER III            |
                                  V                                       V
TIER III   Pathogenic, or pathogenic and toxic effects:             Toxic effects:

           1.  Definitive aquatic animal pathogenicity      Conduct testing according
               tests (§ 154-27); or                         to Subdivision E, and
                                                            proceed with tier testing
           2.  Embryolarvae and life cycle tests            as indicated in Sub-
               (§ 154-28); or                               division E (see § 154-25)

           3.  Aquatic ecosystem-pathogenicity tests
               (§ 154-29); or

           4.  Special tests: tissue culture, micro-   _________________
                organism/stress interaction tests                      |
                          (§ 154-30)                                   |
                               |                                       |
                               V                                       V
                      No adverse effects;                       Adverse effects
                      no further testing                           observed
                           necessary                                   |
                                                                       |
                                                                       V
TIER IV                                                     Simulated or actual field
                                                            tests of aquatic animals
                                                           (see note, below) (§ 154-34)
```

<u>NOTE</u>: Pathogenic effects at TIER III and beyond raise serious questions concerning registration. The Agency will review all data before TIER V testing and decide if a decision concerning registration should be made without further testing.

Figure 8--NONTARGET PLANT TIER TESTING SCHEME FOR MICROBIAL PEST CONTROL AGENTS

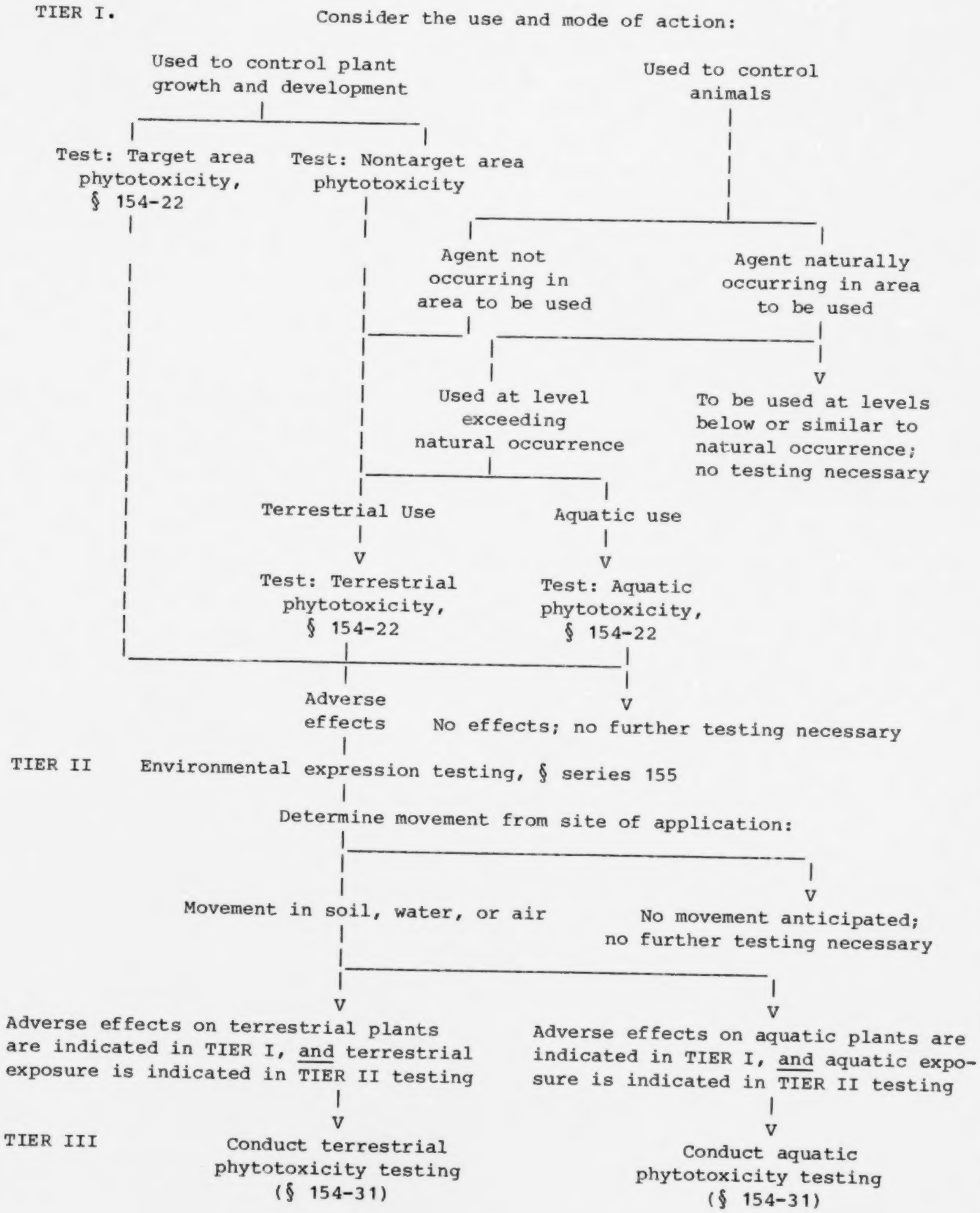

TIER I.
Consider the use and mode of action:
Used to control plant growth and development
Used to control animals
Test: Target area phytotoxicity, § 154-22
Test: Nontarget area phytotoxicity
Agent not occurring in area to be used
Agent naturally occurring in area to be used
Used at level exceeding natural occurrence
To be used at levels below or similar to natural occurrence; no testing necessary
Terrestrial Use
Aquatic use
Test: Terrestrial phytotoxicity, § 154-22
Test: Aquatic phytotoxicity, § 154-22
Adverse effects
No effects; no further testing necessary
TIER II
Environmental expression testing, § series 155
Determine movement from site of application:
Movement in soil, water, or air
No movement anticipated; no further testing necessary
Adverse effects on terrestrial plants are indicated in TIER I, and terrestrial exposure is indicated in TIER II testing
Adverse effects on aquatic plants are indicated in TIER I, and aquatic exposure is indicated in TIER II testing
TIER III
Conduct terrestrial phytotoxicity testing (§ 154-31)
Conduct aquatic phytotoxicity testing (§ 154-31)

Figure 9--NONTARGET INSECT TIER TESTING SCHEME FOR MICROBIAL PEST CONTROL AGENTS: TERRESTRIAL INSECT TESTING WITH NON-GENETICALLY-ENGINEERED MICROORGANISMS

```
TIER I   Test:
         1.  Honey Bee toxicity and pathogenicity test (§ 154-24); and
         2.  Toxicity and pathogenicity tests on three other species of
             beneficial insects from the following groups (no more than
             one species per group):

                  Predaceous hemipterans (§ 154-23)
                  Predaceous coleopterans (§ 154-23)
                  Predaceous mites (§ 154-23)
                  Predaceous neuropterans (§ 154-23)
                  Parasitic hymenopterans (§ 154-23)
                                |
                                V
                     Consider Tier I test results, use pattern,
                     available research and development data on
                      specificity, available fate information
                         |                                  |
                         |                                  |
                         V                                  V
                 Potential toxic and/or             No potential adverse
                  pathogenic effects                  effects observed;
                         |                          no further testing
                         |                               necessary
                         |
                         V
TIER II           Environmental expression testing
                     indicated (§ series 155)
                        |                   |
                        |                   V
                        |         Expression characteristics
                        |             indicate exposure
                        |                     |
                        V                     V
              Expression characteristics    Consider the type of adverse effect
              do not indicate exposure        determined from TIER I testing
                      |                               |                  |
                      |                               |                  |
                      V                               V                  V
             No further testing necessary    Pathogenic effects     Toxic effects
                                                   |                     |
                                                   |                     |
                                                   |                     |
                                                   V                     V
TIER III                                  Consult with Agency     Consult with Agency
                                         regarding simulated or  regarding possible use
                                          actual field testing     restrictions, label
                                         (§§ 154-35 and -36)     precautions, or further
                                           or other testing         testing specific
                                        specific to the problem     to the problem
```

Figure 10--NONTARGET INSECT TIER TESTING SCHEME FOR MICROBIAL PEST CONTROL AGENTS: TERRESTRIAL INSECT TESTING WITH GENETICALLY-ENGINEERED MICROORGANISMS

```
TIERS I AND II
                Conduct appropriate TIER I tests, plus
                TIER II environmental expression tests
                                  |
                                  |
                                  |
                                  V
             Consider use pattern, and available research
                  and development data on host range
                    |                              |
                    |                              |
                    V                              |
 No potential adverse effects;                     V
 no further testing necessary            Potential adverse effects indicated
                                           |                        |
                                           |                        V
                                           |            Expression characteristics
                                           |             do not indicate exposure;
                                           |            no further testing necessary
                                           V
                         Expression characteristics
                            indicate exposure
                                  |
                                  |
                                  |
                                  V
                         Type of adverse effect
                      determined from TIER I testing
                          |                  |
                          |                  |
                          |                  |
                          V                  V
              Pathogenic effects          Toxic effects
                     |                             |
                     |                             |
TIER III             |                             |
                     |                             V
                     |                  Consult with Agency regarding
                     V                  possible use restrictions, label
     Consult with Agency regarding      precautions, or further testing
      simulated or actual field            specific to the problem
     testing (§ 154-35 and -36),
     or other testing specific to
           the problem
```

Table 6-- SUMMARY OF NONTARGET HAZARD GUIDELINES BY PESTICIDE TYPE, TIER, AND NON-TARGET ORGANISM GROUP

	Terrestrial Wildlife	Aquatic Animals	Plants	Beneficial Insects
(1) Biochemicals				
TIER I	§§ 154-6, -7 and § 71-3 (Subdivision E)	§§ 154-8 and -9	§ 154-10 and § 122-1 and -2 (Subdivision J)	§ 154-11
TIER II	Environmental fate tests (§§ 155-1 through -14)			
TIER III	§ 154-12	§ 154-13	§ 154-14	§ 154-15
(2) Microbials				
TIER I	§§ 154-16, -17, and -18	§ 154-19, -20, and -21	§ 154-22	§ 154-23 and -24
TIER II	Environmental expression tests (see §§ 155-15 through -23)			
TIER III	§§ 154-25 and -26	§ 154-25 and -27 through -30	§ 154-31	None
TIER IV	§ 154-33	§ 154-34	None	§ 154-35 and -36

Group A-1: Tier I Testing.

§ 154-6 Avian single dose oral toxicity test: Tier I.

(a) When required. Data on the avian acute oral toxicity of a biochemical pesticide are required by 40 CFR § 158.165 to support the registration of each end-use product intended for outdoor application and each manufacturing-use product that may legally be used to formulate such an end-use product. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Test standards. The data should satisfy the general test standards in § 70-3 of Subdivision E and all of the following test standards:

(1) Test substance. The technical grade of each active ingredient in the product shall be tested.

(2) Species. Testing should be performed on one avian species (preferably bobwhite quail or mallard duck). The species selected should be the same as that selected for the avian dietary toxicity test in § 154-7.

(3) Age. Birds used in this test should be at least 16 weeks old at the beginning of the testing period. Within a given test, all birds should be of the same age.

(4) Controls. A concurrent control group is recommended. If a vehicle (carrier, solvent, or diluent) is used, the concurrent control group should be treated with the vehicle. Vehicles known to be toxic, synergistic, or antagonistic should not be used.

(5) Number of birds per dosage level. Each treatment and control group should contain at least 10 birds. When only one treatment group is tested, at least 30 birds should be tested at that dosage level.

(6) Determination of LD50. The test data must establish:

(i) That the avian single-dose oral LD50 is greater than a maximum test dosage, $\underline{x}$ mg/kg, where $\underline{x}$ is determined by the following equation:

$$\underline{x}\ \text{mg/kg} = 2000\ \text{mg/kg} \times \frac{\text{maximum application rate in grams active ingredient per acre}}{454\ \text{g/acre*}}$$

Only one concurrent vehicle control group and one treatment group dosed at $\underline{x}$ mg/kg may be necessary; or

(ii) A precise LD50 value and corresponding 95 percent confidence interval.

(c) Reporting and evaluation of data. The requirements of § 70-4 and § 71-1(c) of Subdivision E apply for all products to be tested in accordance with this section.

(d) Tier progression. (1) Testing at Tier II is required by 40 CFR § 158.165 if any one or more of the following occur:

(i) The maximum expected environmental concentration is equal to or greater than 1/5 the avian single-dose oral LD50 value expressed in ppm. The LD50 in mg/kg is converted to ppm by the following formula:

$$\text{ppm} = \text{LD50} \div \frac{\text{average daily food consumption (g)}}{\text{body weight (g)}}$$

(ii) Signs of abnormal behavior are observed in the avian singledose oral toxicity test at levels equal to or less than the maximum expected environmental concentration;

(iii) Growth, development, or reproductive effects may be expected, based on observed effects in the avian singledose oral toxicity test, available environmental fate data, use pattern information, and results of tests required to support human safety (Subdivision F).

(2) If none of the criteria in paragraph (d)(1) of this section are met, then additional testing at Tier II [environmental fate testing (§§ 155-1 through -14)] is not necessary.

(e) Reference: test protocol. An example of an acceptable protocol for conducting an avian single-dose oral toxicity test is provided in § 71-1 of Subdivision E.

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*(Number of grams per pound. One pound is a typical application rate for conventional pesticides.)

§ 154-7 Avian dietary toxicity test: Tier 1.

(a) When required. Data on the avian dietary toxicity of a biochemical pesticide are required to support the registration of each end-use product that is not highly volatile (estimated volatility greater than $5x10^{-5}$ atm. m^3/mol.) and that is intended for outdoor application, and each manufacturing-use product that may be used to formulate such an end-use product. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Test standards. Data should satisfy the general test standards in § 70-3 of Subdivision E and all of the following test standards:

(1) Test substance. The technical grade of each active ingredient in the product shall be tested.

(2) Species. Testing should be performed on one avian species (preferably bobwhite quail or mallard duck). The species selected should be the same as that selected for the avian single-dose oral toxicity test required by § 154-6.

(3) Age. Birds used in this study should be from 10 to 17 days old at the beginning of the testing period. Within a given test, all birds should be of the same age.

(4) Controls. A concurrent control group is required. If a vehicle (carrier, solvent, or diluent) is used, the concurrent control group should be treated with the vehicle. Vehicles known to be toxic, synergistic, or antagonistic should not be used.

(5) Number of birds per concentration level. Each treatment and control group should contain at least 10 birds. When only one treatment group is tested, at least 30 birds should be tested at that treatment level.

(6) Determination of LC50. The test data must establish:

(i) That the 8 day dietary (5 days treated diet and 3 days untreated diet) LC50 is greater than a maximum test concentration, x ppm, where x ppm is determined by the following equation:

$$\underline{x} \text{ ppm} = 5000 \text{ ppm} \times \frac{\text{maximum application rate in grams active ingredient per acre}}{454/\text{acre}^*}$$

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*Number of grams per pound. One pound is a typical application rate for conventional pesticides.

Only one concurrent vehicle control group and one treatment group fed a concentration equal to x ppm may be necessary; or

(ii) A precise LD50 value and corresponding 95 percent confidence intervals.

(c) Reporting and evaluation of data. The provisions of § 70-4 and § 71-2(c) of Subdivision E apply for all products to be tested in accordance with this section.

(d) Tier progression. (1) Testing at Tier II is required by 40 CFR § 158.165 if any one or more of the following occur:

(i) The maximum expected environmental concentration is equal to or greater than 1/5 the avian dietary LC_{50} value

(ii) Signs of abnormal behavior are observed in the avian dietary toxicity test at levels equal to or less than the maximum expected environmental concentration;

(iii) Growth, development, or reproductive effects may be expected, based on observed effects in the avian dietary toxicity test, available fate data, use pattern information, or results of tests required to support human safety (Subdivision F).

(2) If none of the criteria in paragraph (d)(1) of this section is met, then additional testing at Tier II, Environmental Fate Testing (§§ 155-1 through -14), is not necessary.

(e) Reference: test protocol. An example of an acceptable protocol for conducting an avian dietary toxicity test is provided in § 71-2 of Subdivision E and as required by 40 CFR 158.145.

§ 154-8 Freshwater fish acute bioassay: Tier I.

(a) When required. (1) Toxicity (or toxic-like effects) data on a freshwater fish are required by 40 CFR § 158.165 to support the registration of each end-use product intended for outdoor terrestrial application and each manufacturing-use product that legally may be used to formulate such an end-use product. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(2) If the pesticide will be introduced directly into an aquatic environment when used as directed, then it must be tested as indicated in §§ 72-1 through -7 of Subdivision E, and as required by 40 CFR § 158.145.

(b) Test standards. The general and specific test standards for biochemical pesticides are the same as those set forth in Subdivision E in §§ 70-3 and 72-1, respectively, with the following exceptions:

(1) Testing should be performed on one species, preferably rainbow trout; and

(2) One maximum hazard concentration may be tested in lieu of conducting a definitive LC_{50} or EC_{50} test if exposure of 30 organisms to a concentration of 1,000 times the expected aquatic environmental concentration or 100 mg/l of water (whichever is greater) produces less than 50 percent mortality.

(c) Reporting of data. The reporting provisions are the same as those set forth in Subdivision E of the guidelines (§ 72-1).

(d) Tier progression. (1) Biochemical agents that meet one of the following three criteria will not require testing at Tier II [environmental fate (§§ 155-1 through -14)] except as noted in paragraph (d)(2) below:

(ii) The amount of biochemical applied is small (less than 0.7 ounces (20 grams) active ingredient per acre per application); or

(iii) The estimated volatility of the biochemical is high (equal to or greater than $5x10^{-5}$ atm. m^3/mol).

(2) Biochemical agents that meet one or more of the above three criteria of paragraph (d)(1) of this section require testing at Tier II [environmental fate (§§ 155-1 through -14)] if any of the following occur:

(i) Signs of abnormal behavior are reported in the Tier I test at concentrations less than or equal to the maximum expected concentration in water; or

(ii) The maximum expected concentration in water is equal to or greater than 0.1 the LC_{50} determined by testing outlined in this section; or

(iii) The maximum expected concentration in water is equal to or greater than 0.01 the LC_{50} determined by testing outlined in this section and adverse effects on growth, develdevelopment, or reproduction may be expected, based on Tier I test data (§ series 154), available environmental fate data (e.g., from the product's research and development), use pattern information, or available effects data on phylogenetically similar target species.

(3) If the criteria in paragraph (d)(1) of this section are not met, then additional testing at Tier II (§§ 155-1 through -14) is required.

(e) References. (1) Examples of acceptable protocols for conducting a freshwater fish acute toxicity study may be found in the following references. Fish species listed in reference (ii) are acceptable, with the exception of goldfish:

(i) ASTM Standard E 729-80, Practice for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians. American Society for Testing and Materials, 1916 Race Street, Philadelphia, PA 19103.

(ii) Committee on Methods for Toxicity Tests with Aquatic Organisms. 1975. Methods for Acute Toxicity Tests with Fish, Macroinvertebrates and Amphibians. U.S. Environmental Protection Agency, Ecol. Res. Series, EPA 660/3-75-009. 61 pp.

(2) The following may contain useful background information for developing acceptable protocols:

(i) Weber, C.E. (ed.). 1973. Biological field and laboratory methods for measuring the quality of surface waters and effluents. U.S. Environmental Protection Agency, Environ. Monit. Series, EPA 670/4-73-001.

(ii) Anonymous. 1975. Standard Methods for the Examination of Water and Wastewater. 14th Ed. American Public Health Assoc. Washington, D.C.

§ 154-9 Freshwater aquatic invertebrate acute bioassay: Tier I.

(a) When required. (1) Toxicity (or toxic-like effects) data on a freshwater aquatic invertebrate are required by 40 CFR § 158.165 to support the registration of each end-use product intended for outdoor terrestrial application and each manufacturing-use product that legally may be used to formulate such an end-use product. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(2) If the pesticide will be introduced directly into an aquatic environment when used as directed, then it must be tested as indicated in §§ 72-1 through -7 of Subdivision E and as required by 40 CFR § 158.145.

(b) Test standards. The general and specific test standards for biochemical pesticides are the same as those set forth in Subdivision E in §§ 70-3 and 72-1, respectively, with the following exception: One maximum hazard concentration may be tested in lieu of conducting a definitive LC_{50} or EC_{50} test if exposure of 30 organisms to a concentration of 1,000 times the expected aquatic environmental concentration or 100 mg/l of water (whichever is higher) produces less than 50 percent mortality.

(c) Reporting of data. The provisions for reporting of data are the same as those set forth in Subdivision E of the guidelines (§ 72-2).

(d) Tier progression. (1) Biochemical agents that meet one or more of the following three criteria will not require testing at Tier II, [Environmental Fate (§§ 155-1 through -14)] except as noted in paragraph (d)(2) below:

(i) The use pattern or formulation precludes the possibility of significant exposure of aquatic animals when the pesticide is used as directed; or

(ii) The amount of biochemical applied is small (less than 0.7 ounces (20 grams) active ingredient per acre); or

(iii) The estimated volatility of the biochemical is high (equal to or greater than 5×10^{-5} atm. m^3/mol).

(2) Biochemical pesticides that meet one or more of the above three criteria require testing at Tier II [environmental fate (§§ 155-1 through -14)] if any of the following occur:

(i) Signs of abnormal behavior are reported in the Tier I test at concentrations less than or equal to the maximum expected concentration in water; or

(ii) The maximum expected concentration in water is equal to or greater than 0.1 the LC_{50} determined by testing outlined in this section; or

(iii) The maximum expected concentration in water is equal to or greater than 0.01 the LC_{50} determined by testing outlined in this section, and adverse effects on growth, development, or reproduction may be expected, based on Tier I test data, available environmental fate data (e.g., from the product's research and development), use pattern information, or available effects data on phylogenetically similar target species.

(3) If the criteria in paragraph (d)(1) of this section are not met, then additional testing at Tier II (§§ 155-1 through -14) is required.

(e) References. (1) Examples of acceptable protocols for conducting a freshwater aquatic invertebrate acute toxicity study may be found in the following reference. Aquatic invertebrate test temperatures found in reference (ii) are acceptable with the exception of 17°C for *Daphnia spp.* *Daphnia* should be tested at 20° ± 1°C.

(i) ASTM Standard E 729-80, Practice for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphiobians. American Society for Testing and Materials, 1916 Race Street, Philadelphia, PA 19103.

(ii) Committee on Methods for Toxicity Tests with Aquatic Organisms. 1975. Methods for Acute Toxicity Tests with Fish, Macroinvertebrates and Amphibians. U.S. Environmental Protection Agency, Ecol. Res. Series, EPA 660/3-75-009. 61 pp.

(2) The following may contain useful background information for developing acceptable protocols:

(i) Weber, C.E. (ed.). 1973. Biological field and laboratory methods for measuring the quality of surface waters and effluents. U.S. Environmental Protection Agency, Environ. Monit. Series, EPA 670/4-73-001.

(ii) Anonymous. 1975. Standard Methods for the Examination of Water and Wastewater. 14th Ed. American Public Health Assoc., Washington, D.C.

§ 154-10 Plant studies: Tier I.

(a) When required. (1) Data on the toxic effects of a biochemical pesticide on plant growth and development are required as specified by 40 CFR § 158.165 to support the registration of each end-use product intended for outdoor application and each manufacturing-use product that legally may be used to formulate such an end-use product. [See § 120-1(e) of Subdivision J.] See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional dicussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(2) When plant studies are required as outlined in paragraph (a)(1) of the section, the indicated tests (in Subdivision J) should be conducted for the following use patterns:

(i) Plant-controlling biochemical pesticides. The target area phytotoxicity test (§ 121-1), the seed germination/seeding emergence and vegetative vigor tests (§ 122-1) and the growth and reproduction of aquatic plant tests (§ 122-2) should be performed.

(ii) Animal-controlling biochemical pesticides. The seed germination/seedling emergence and vegetative vigor tests (§ 122-2) should be performed except where:

(A) The material is to be used in a contained manner rather than in a general broadcast or band manner;

(B) The material is to be used as a broadcast treatment at levels less than three times the naturally-occurring level; or

(C) The material occurs naturally in the plant community type where usage of the product is intended.

(b) Test standards. The phytotoxicity studies as outlined in this subdivision should meet the Subdivision J general test standards (§ 120-3) and specific test standards [§ 121-1(b), 122-1(b), and 122-2(b)] for the appropriate tests with the following exceptions.

(1) Test substance. A typical end-use product shall be tested.

(2) Dose levels. One concentration level equal to not less than the maximum label rate should be tested where the active ingredient application solution concentration is 10 ppb or greater. The phrase "the maximum label rate" means the amount of active ingredient that may be used per land area or applied directly to the surface of a 15-cm or 6-inch column of water.

(c) Reporting. In addition to the general information required in § 120-4(b) of Subdivision J, the reporting requirements for the other tests [§§ 121-1(c), 122-1(c), and 122-2(c)] should be followed.

(d) Tier progression. (1) If an adverse effect or response on plant growth and development for any terrestrial plant species is 25 percent or greater with respect to the control or 50 percent or greater for aquatic plants, then testing at Tier II (Environmental Fate, §§ 155-1 through 155-14) is required as specified in 40 CFR § 158.165.

(2) If less than a 25 percent adverse effect or response is noted for terrestrial plants or 50 percent for aquatic plants no additonal testing at higher tiers is ordinarily necessary. The

Agency, however, after reviewing the data, may require certain additional tests to determine a more accurate no observed effect level.

§ 154-11 Nontarget insect testing: Tier I.

(a) When required. (1) General. Tier I testing for effects of biochemical pesticides on nontarget insects is required on a case-by-case basis as specified by 40 CFR § 158.165 to support the registration of each end-use product, and of each manufacturing-use product that legally may be used to formulate such an end-use product.

(2) Tests required. (i) Terrestrial insects. The registrant must report any adverse effects noted during efficacy testing (§ 156-2), and/or any data that indicate potential for adverse effects on nontarget insects.

(ii) Aquatic insects. (A) If the biochemical is introduced directly into an aquatic environment when used as directed, testing must be conducted in accordance with Subdivisions E and L.

(B) If the biochemical is not introduced directly into an aquatic environment when used as directed, testing must be conducted as specified in § 154-9.

(b) Test standards. Studies conducted in accordance with paragraph (a)(2)(ii)(A) should meet the applicable requirements outlined in §140-3 of Subdivision L. Studies conducted in accordance with paragraph (a)(2) (ii)(B) should meet the applicable requirements outlined in §§ 150-3 and 154-9 of this subdivision.

(c) Reporting and evaluation of data. The test report should contain the information required in Subdivision L (§ 140-4 and other applicable sections) or this subdivision (§ 150-4 and other applicable sections), as appropriate.

(d) Tier progression. (1) Terrestrial insects. Tier II testing (§ series 155) is required by 40 CFR § 158.165 according to the following criteria:

(i) If adverse effects are noted during efficacy testing (§ 156-2), or

(ii) If other data exist which indicated potential for adverse effects on nontarget terrestrial insects.

(2) If neither of the criteria in paragraph (d)(1)(i) or (ii) of this section apply, no further testing is necessary.

(3) Aquatic insects. (i) If the biochemical is introduced directly into an aquatic environment when used as directed, testing is required as outlined in Subdivisions E and L.

(ii) If the biochemical is not introduced directly into an aquatic environment when used as directed, testing is required as specified in § 154-9.

Group A-2: Tier III Testing.

§ 154-12 Terrestrial wildlife testing: Tier III.

(a) When required. Data on the effects of a biochemical pest control agent on terrestrial wildlife are required by 40 CFR § 158.165 as outlined in section series 71 of Subdivision E to support the registration of each end-use product intended for outdoor application and each manufacturing-use product that legally may be used to formulate such an end-use product, if:

(1) Environmental fate characteristics indicate that the estimated concentration of the biochemical pesticide in the terrestrial environment is equal to or greater than 1/5 the avian dietary LC50 or the avian single dose oral LD50 (converted to ppm); or

(2) The pesticide or any of its metabolites or degradation products are stable in the environment to the extent that potentially toxic amounts may persist in the avian feed.

(3) See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Test standards. The test standards in § 70-3 and §§ 71-2 through -5 of Subdivision E apply.

(c) Reporting and evaluation of data. The reporting and evaluation provisions in § 70-4 and §§ 71-2 through -5 of Subdivision E apply.

(d) Tier progression. Further testing shall be conducted as specified in §§ 71-2 through -5 of Subdivision E.

§ 154-13 Aquatic animal testing: Tier III.

(a) When required. Data on the 40 CFR § 158.165 effects of a biochemical pest control agent on aquatic animals are required to support the registration of each end-use product intended for outdoor application and each manufacturing-use product, that legally may be used to formulate such an end-use product if environmental fate characteristics indicate that the estimated environmental concentration of the biochemical agent in the aquatic environment is equal to or greater than 0.01 of any EC50 or LC50 determined in testing outlined by § 154-8 or -9. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Test standards. The test standards in § 70-3 and §§ 72-1 through -7 of Subdivision E apply.

(c) Reporting and evaluation of data. The reporting and evaluation provisions in § 70-4 and §§ 72-1 through -5 of Subdivision E apply.

(d) Tier progression. Further testing shall be conducted as outlined in §§ 72-1 through -7 of Subdivision E and required by 40 CFR § 158.165.

§ 154-14 Plant studies: Tier III.

(a) When required. Data on the effects of a biochemical pest control agent on plant growth and development are required by 40 CFR § 158.165 on a case-by-case basis to support the registration of each end-use product agent intended for outdoor application and each manufacturing-use product that legally may be used to formulate such a product where the material may be moved from the site of application by air, soil, or water. The extent of movement will be determined by the Tier II environmental fate tests (see § series 155). [See § 120-1(e) of Subdivision J.] Refer 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Test standards. The test standards in Tiers II through III (§ 123-1 through § 124-2) of Subdivision J apply.

(c) Reporting. The reporting provisions in Tiers II through III [§ 123-1 through § 124-2] of Subdivision J apply.

(d) Tier progression. The tier progression criteria in Tiers II through III [§ 123-1 through § 124-2] of Subdivision J apply.

§ 154-15 Nontarget insect testing: Tier III.

(a) When required. Data on the effects of a biochemical pest control agent on nontarget insects are required by 40 CFR § 158.165 to support the registration of each end-use product intended for outdoor application and each manufacturing-use product that legally may be used to formulate such an end-use product, when results of Tier I tests (§ 154-11) indicate potential adverse effects on nontarget insects and results of Tier II tests (section series 155) indicate exposure of nontarget insects. Tier III testing should only be performed following consultation with the Agency.

(b) Test standards. The test standards in § 140-3 of Subdivision L apply.

(c) Reporting and evaluation of data. The reporting and evaluation requirements in § 140-4 of Subdivision L apply.

(d) Tier progression. None.

Subseries 154B: MICROBIAL AGENTS

Group B-1: Tier I Testing.

§ 154-16 Avian single-dose oral toxicity and pathogenicity test: Tier I.

(a) When required. Data on the avian acute oral toxicity of a microbial pest control agent are required by 40 CFR § 158.165 to support the registration of each end-use product intended for outdoor application and each manufacturinguse product that legally may be used to formulate such an end-use product. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Test standards. Data should be derived from tests that satisfy the general test standards in § 150-3 of this subdivision and the following test standards:

(1) Test substance. The technical grade of each active ingredient in the product shall be tested.

(2) Species. Testing should be performed on one avian species (preferably bobwhite quail or mallard duck).

(3) Age. Birds used in this test should be from 10 to 17 days old at the beginning of the test period. Within a given test, all birds should be of the same age.

(4) Controls. (i) A negative control group is necessary;

(ii) A concurrent control group is recommended and should be treated with the technical grade of the active ingredient containing inactivated microbial agent.

(5) Number of birds per dosage level. Each treatment and control should contain at least 10 birds. When only one treatment group is tested, at least 30 birds should be tested at that level.

(6) Maximum hazard dosage level. The highest dosage level tested should not be less than 10 times the adjusted host equivalent amount. If the 10x amount is not feasible, a 5x or 2x amount should be tested. A reason shall be provided to support any reduction in the highest dosage level from 10 times the adjusted host equivalent amount.

(7) Determination of an LD50. The test data must establish that the avian single-dose oral LD50 or ED50 is greater than the maximum hazard dosage level, or must establish an LD50 or ED50 value and corresponding 95 percent confidence intervals, if possible.

(8) Duration of test. Control and treated groups should be observed for at least 30 days after dosing.

(c) Reporting and evaluation of data. In addition to the information specified in § 150-4 of this subdivision, the test report should contain the following information:

(1) Age of the birds tested;

(2) Mean body weights for each test and control group at weekly intervals;

(3) Diet used;

(4) Pen dimensions;

(5) Ambient temperature and humidity; (6) Photoperiod and lighting;

(7) Total feed consumption for each test and control group at weekly intervals;

(8) Preparation of test material;

(9) Amount of test material dosed per bird;

(10) Amount of vehicle dosed per bird, if a vehicle is used;

(11) Number of birds per treatment level;

(12) Number of controls used;

(13) LD50 or ED50 in mg/kg, with 95 percent confidence limits, if obtained;

(14) Methods used for calculation of LD50;

(15) Slope of the dose response line, if obtained;

(16) Time and date of mortalities;

(17) All signs of intoxication, abnormal behavior, and regurgitation (if any occurs);

(18) Reports of any pathogenic effects or pathological changes;

(19) Results of gross necropsies conducted on enough birds to characterize any gross lesions; and

(20) Reasons to support a reduction (if any) in the highest dosage level required by paragraph (b)(6) of this section.

(d) *Tier progression*. (1) If any toxic or pathogenic effects are observed at the maximum hazard dosage level in this study, testing at Tier II, environmental expression testing (§§ 155-15 through -20), is required as specified by 40 CFR § 158.165.

(2) If toxic or pathogenic effects are not observed in this study, additional testing at higher tiers is ordinarily not necessary.

(e) *References*. The following references are provided for use in the development of acceptable test protocols for conducting an avian single dose oral toxicity test with a microbial pest control agent:

(1) Ignoffo, C.M. 1973. Effects of entomopathogens on vertebrates. *Annals N.Y. Acad. Sci.* 217:141-164.

(2) Lautenschlager, R.A., and J.D. Podgwaite. 1979. Passage of nucleopolyhedrosis virus by avian and mammalian predators of the gypsy moth, *Lymantria dispar*. *Environ. Entomol.* 8(2):210-214.

(3) Friend, M., and D.O. Trainer. 1971. Experimental duck virus hepatitis in the mallard. *Avian Disease* 16(4): 692-699.

(4) Narayanan, K., G. Santharam, S. Easwaramoorthy, and S. Jayaraj. 1978. Lack of susceptibility of poultry birds to nuclear polyhedrosis virus of groundnut red-hairy caterpillar, *Amsacta albistriga* (W.). *Indian J. Exper. Biol.* 16(12):1322-1324.

(5) Podgwaite, J.D., and R.R. Galipeau. 1978. Effects of nucleopolyhedrosis virus on two avian predators of the gypsy moth. USDA, For. Serv. Res. Note, NE - 251, 2 pp.

(6) Summers, M., R. Engler, L.A. Falcon, and P. Vail, eds. 1975. Guidelines for Safety Testing of Baculoviruses, Pp. 179-184 *in* Baculoviruses for Insect Pest Control. Safety Considerations. American Society for Microbiology, Washington, D.C.

(7) Tucker, R.K., and M.A. Haegele. 1971. Comparative acute oral toxicity of pesticides to six species of birds. *Toxicol. Appl. Pharmacol.* 10:57-65 (see pp. 57-59).

(8) Wolf, K. 1975. Evaluation of the exposure of fish and wildlife to nuclear polyhedrosis and granulosis viruses. Pp. 109-111 in Baculoviruses for Insect Pest Control. Safety Considerations. American Society for Microbiology, Washington, D.C.

§ 154-17 Avian injection pathogenicity test: Tier I.

(a) When required. Data on the avian acute injection pathogenicity of a microbial pest control agent are required by 40 CFR § 158.165 to support the registration of each end-use product intended for outdoor application and each manufacturing-use product that legally may be used to formulate such an end-use product. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Test standards. Data sufficient to satisfy the requirement in paragraph (a) of this section should be derived from tests which satisfy the purposes of the general test standards in § 150-3 of this subdivision, and all the following test standards:

(1) Test substance. Data should be derived from testing conducted with the technical grade of each active ingredient in the product.

(2) Species. Testing should be performed on one avian species (preferably bobwhite quail or mallard duck).

(3) Age. Birds used in this test should be from 10 to 17 days old at the beginning of the testing period. Within a given test, all birds shall be the same age.

(4) Controls. (i) A negative control group is necessary;

(ii) A concurrent control group is necessary and should be treated with the technical grade of the active ingredient containing inactivated microbial agent.

(iii) Two untreated contact control birds are recommended, and should be placed in with the treatment group receiving the maximum hazard dosage.

(5) Number of birds per dosage level. Each treatment and control group should contain at least 10 birds. When there is only one treatment group at least 30 birds should be tested at that treatment level.

(6) Route of exposure. The test material should be administered by intraperitoneal or intravenous injection. The intravenous route may not be feasible because of problems related to the size of particles in the inoculum or technical problems related to the age or size of the birds.

(7) Maximum hazard dosage level. The highest dosage level tested should not be less than an amount equal to one adjusted host equivalent. If this amount is not feasible, a 1/2x, 1/5x or 1/10x amount should be tested. A reason shall be provided to support any reduction in the highest dosage level from an amount equal to the adjusted host equivalent amount.

(8) Determination of an ED50. The test data must establish that the avian injection ED50 is greater than the maximum hazard dosage level, or sequentially lower levels shall be tested to provide a dose-response series which includes at least one level in which no mortality occurs. If possible, an ED50 value and corresponding 95% confidence intervals shall be established.

(9) Duration of test. Control and treated birds should be observed for at least 30 days after dosing.

(c) Reporting and evaluation of data. In addition to the information specified in § 150-4 of this subdivision, the test report should contain the following information:

(1) Age of the birds tested;

(2) Mean body weights for each test and control group at weekly intervals;

(3) Diet used;

(4) Pen dimensions;

(5) Ambient temperature and humidity;

(6) Photoperiod and lighting;

(7) Total feed consumption for each test and control group at weekly intervals;

(8) Preparation of test material;

(9) Amount of test material injected per bird;

(10) Amount of vehicle injected per bird, if vehicle is used;

(11) Number of birds per treatment level;

(12) Number of controls used;

(13) Time and date of mortalities;

(14) ED50 in mg/kg, with 95% confidence limits, if obtained;

(15) Results of gross necropsy conducted on all birds dying before termination of the test and on a representative sample of those that survived, and on the two contact control birds. The necropsy report should include any evidence of multiplication of microbes (e.g. lesions), at:

(i) The site of injection; and

(ii) Distant sites including liver, kidney, lungs, spleen, cerebrospinal system, gastrointestinal system, and circulatory system;

(16) A description of the methods used to assess the cause and effects of any lesions noted where there is evidence that the microbial agent is multiplying in the bird;

(17) Assessment of any effects noted; and

(18) Reason to support reduction in highest dosage section.

(d) Tier progression. (1) If any pathogenic effects are observed at the maximum hazard dosage level in this study, testing at Tier II [environmental expression testing (§§ 155-15 through -20)] is required as specified in 40 CFR § 158.165.

(2) If no pathogenic effects are observed in this study, no additional testing at higher tiers ordinarily is necessary.

(e) References. The following references are provided for use in the development of acceptable test protocols for conducting an avian injection pathogenicity test with microbial pest control agents:

(1) Ahmed, M.M., and D.H. Walker. 1979. The metabolism of DDT in vivo by the Japanese quail (*Coturnix coturnix japonica*). *Pesticide Biochem. Physiol.* 10:40-48.

(2) Friend, M., and D. O. Trainer. 1974a. Experimental DDT-Duck hepatitis virus interaction studies. *J. Wildl. Manage.* 38(4):887-895.

(3) __________ . 1974b. Experimental Dieldrin-Duck hepatitis virus interaction studies. *J. Wildl. Manage.* 38(4):896-902.

(4) ________ . 1972. Duck hepatitis virus interaction with DDT and Dieldrin in adult mallards. *Bull. Environ. Contam. Toxicol.* 7(4):202-206.

(5) Friend, M., and D.O. Trainer. 1971. Experimental duck virus hepatitis in the mallard. *Avian Disease* 16(4):692-699.

(6) ________ . 1970. Polychlorinated biphenyl: interaction with duck hepatitis virus. *Science* 170(3964):1314-1316.

(7) Summers, M., R. Engler, L.A. Falcon, and P. Vail, eds. 1975. Pp. 179-184 *in* Guidelines for Safety Testing of Baculoviruses. Baculoviruses for Insect Pest Control: Safety Considerations. American Society for Microbiology, Washington, D.C.

§ 154-18 *Wild mammal toxicity and pathogenicity testing: Tier I*.

(a) *When required*. Data on wild mammal toxicity and pathogenicity may be required by 40 CFR § 158.165 on a case-by-case basis to support the registration of end-use products intended for outdoor application and manufacturing-use products that legally may be used to formulate such end-use products. The toxicity and pathogenicity data outlined in Subdivision F for evaluating hazard to humans and domestic animals are normally adequate to indicate hazard to wild mammals. Under certain conditions, however, these data are not sufficient to assess the potential hazard to wild mammals likely to be exposed to a microbial pest control agent. An example of one circumstance when such data may be required is the situation in which data indicate that there is considerable variation in sensitivity of different mammalian species to the effects of a microbial pest control agent, and there is evidence that wild mammals are heavily exposed to microbial pest control agents. See 40 CFR § 158.50 and §158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) *Test standards*. Data should be derived from tests that satisfy the general test standards in § 150-3 of this subdivision and the following test standards:

(1) *Test substance*. Data shall be derived from testing conducted with the technical grade of each active ingredient in the product.

(2) *Species*. Testing should be performed on a mammalian species representative or indicative of those found in the area(s)

likely to be affected by the proposed use pattern(s). Test animals may be reared in pens or captured in the wild, and should be phenotypically indistinguishable from wild mammals. Endangered or threatened animals shall not be used.

(3) Controls. (i) A negative control group is necessary; and

(ii) A concurrent control group is necessary and should be treated, when possible, with the technical grade of the active ingredient containing inactivated (e.g., autoclaved) microbial agent.

(4) Route of exposure. The test material should be administered by gavage (acute oral dose) or by injection (intraperitoneal or intravenous). The route shall be determined after consultation with the Agency.

(5) Maximum hazard dosage level. The standards for maximum hazard dosage level, determination of an ED50, and duration of test that are found in the avian single dose oral toxicity and pathogenicity test § 154-16(b) and the avian injection pathogenicity test § 154-17(b) apply also to the respective tests on mammals.

(c) Reporting and evaluation of data. In addition to the information specified in § 150-4 of this subdivision, test reports should contain the same information required for the avian single-dose oral toxicity and pathogenicity test § 154-16(c) and the avian injection pathogenicity test § 154-17(c), adapted appropriately for mammalian test procedures.

(d) Tier progression. (1) If any toxic or pathogenic effects on mammalian species are observed at the maximum hazard dosage level in this study, testing at Tier II [environmental expression testing (§§ 155-15 through -20)] is required as specified by 40 CFR § 154.165.

(2) If toxic or pathogenic effects are not observed in this study, additional testing at higher tiers is ordinarily not necessary.

(e) References. The following references are provided for use in the development of acceptable test protocols for conducting wild mammal toxicity and pathogenicity tests with microbial pest control agents:

(1) Agr. Res. Service, USDA Animal Disease and Parasite Research Division. 1969. The toxicity of some organic herbicides to cattle, sheep, and chickens. A.R.S. Production Research Report No. 106.

(2) Barnes, R.W., C.F. Meincke, W.C. McLane, and C.S. Rehnborg. 1970. Long-term feeding and other toxicitypathogenicity

studies on rats using a commercial preparation of the nuclear-polyhedrosis virus of Heliothis zea. J. Invert. Pathol. 16:112-115.

(3) Fisher, R., and L. Rosner. 1959. Toxicology of the microbial insecticide, Thuricide. Agric. Food Chem. 7(10):686-688.

(4) Ignoffo, C.M. 1973. Effects of entomopathogens on vertebrates. Annals N.Y. Acad. Sci. 217:141-164.

(5) Ignoffo, C.M. 1971. Intraperitoneal injection of white mice with nucleopolyhedrosis virus of the beet armyworm, Spodoptera exigua. J. Invert. Pathol. 17(3):453-454.

(6) Ignoffo, C.M., W.M. Barker, and C.W. McCoy. 1973. Lack of per os toxicity or pathogenicity in rats fed the fungus, Hirsutella thompsonii. Entomophaga 18(3):333-335.

(7) Ignoffo, C.M., C. Garcia, R.W. Kapp, and W.B. Coate. 1975. An evaluation of the risks to mammals of the use of an entomopathogenic fungus, Nomuraea rileyi, as a microbial insecticide. In: Baculoviruses for Insect Pest Control: Safety Considerations. Selected papers from EPA/USDA Working Symposium, Amer. Soc. Microbiology, Washington, D.C.

(8) Ignoffo, C.M., and A.M. Heimpel. 1965. The nuclear polyhedrosis virus of Heliothis zea (Boddie) and Heliothis virescens (Fabricus) Part V. Toxicity-pathogenicity of virus to white mice and guinea pigs. J. Invert. Pathol. 7:329-340.

(9) Ignoffo, C.M., J.J. Petersen, H.C. Chapman, and J.F. Novotny. 1974. Lack of susceptibility of mice and rats to the mosquito nematode, Reesimermis nielseni, Tsai and Grundmann. Mosquito News 34(4):425-428.

(10) Lamanna, C., and L. Jones. 1963. Lethality for mice of vegetative and spore forms of Bacillus cereus and Bacillus cereus-like insect pathogens injected intraperitoneally and subcutaneously. J. Bacteriology 85:532-535.

(11) Lautenschlager, R.A., C.H. Kircher, and J.D. Podgwaite. 1977. Effect of nucleopolyhedrosis virus on selected mammalian predators of the gypsy moth. USDA, For. Serv. Res. Paper, NE-377, 6p.

(12) Lautenschlager, R.A., and J.D. Podgwaite. 1979. Passage of nucleopolyhedrosis virus by avian and mammalian predators of the gypsy moth, Lymantria dispar. Environ. Entomol. 8(2):210-214.

(13) Lautenschlager, R.A., and J.D. Podgwaite. 1977. Passage of infectious nuclear-polyhedrosis virus through the alimentary

tracts of two small mammal predators of the gypsy moth, Lymantria dispar. Environ. Entomol. 6(5):737-738.

(14) Meinacke, C.F., W.C. McLane, and C.S. Rehnborg. 1970. Toxicitypathogenicity studies of a nuclear-polyhedrosis virus of Heliothis zea in white mice. J. Invert. Pathol. 15:10-14.

(15) Pounds, J.G. 1977. Safety and potential hazards of the entomopathogen Mattesia trogodermae to non-target species. Ph.D. Thesis, University of Wisconsin, Madison, Wisconsin. 131 pp.

(16) Summers, M., R. Engler, L.A. Galcon, and P. Vail, eds. 1975. Pp. 179-184 in Guidelines for Safety Testing of Baculoviruses for Insect Pest Control: Safety Considerations. American Society for Microbiology, Washington, D.C.

(17) Smirnoff, W.A., and C.F. MacLeod. 1964. Apparent lack of effects of orally introduced polyhedrosis virus on mice and of pathogenicity of rodent-passed virus for insects. J. Insect Pathol. 6:537-538.

(18) Watts, D.M., R.F. Tammariello, J.M. Dalrymple, B.F. Eldridge, P.K. Russell, and F.H. Top, Jr. 1979. Experimental infection of vertebrates of the Pocomoke Cypress Swamp, Maryland with Keystone and Jamestown Canyon viruses. Am. J. Trop. Med. Hyg. 28(2):344-350.

(19) Webb, R.E., and H. Horsfall, Jr. 1967. Endrin resistance in the pine mouse. Science 156:1862.

(20) Wolf, K. 1975. Evaluation of the exposure of fish and wildlife to nuclear polyhedrosis and granulosis viruses. Pp. 109-111 in Baculoviruses for Insect Pest Control: Safety Considerations. American Society for Microbiology, Washington, D.C.

§ 154-19 Freshwater fish toxicity and pathogenicity testing: Tier I.

(a) When required. Data on pathogenicity and/or toxicity to fish are required by 40 CFR § 158.165 to support the registration of each end-use product intended for outdoor application and each manufacturing-use product that legally may be used to formulate such an end-use product.

(b) Test standards. Data should be derived from tests that satisfy the general test standards in § 150-3 of this subdivision, and the following test standards:

(1) Test substance. (i) Data to support the registration of an end-use formulated product or a manufacturinguse product shall be derived from testing conducted with:

(A) The technical grade of each active ingredient in the product when the test substance is added directly to the test water; and

(B) The most challenging form (in terms of pathogenicity and toxicity) of each active ingredient (microorganism) in the product when the test substance is administered in the diet or by injection.

(ii) In addition, data from testing with the end-use product are required to support the registration of any end-use product if an ingredient in the end-use product other than the active ingredient is expected to:

(A) Enhance the toxicity or pathogenicity of the active ingredient; or

(B) Enhance the ability of the active ingredient (microorganism) to survive or replicate in an aquatic environment; or

(C) Independently cause toxicity to aquatic organisms.

(2) Test organisms. (i) Testing should be performed on one or two fish species depending upon the site of pesticide application as follows:

(A) Terrestrial application: test one species, preferably rainbow trout.

(B) Direct application into a freshwater environment: test two species, preferably rainbow trout and bluegill sunfish.

(C) Direct application into an estuarine or marine environment: test one species, preferably rainbow trout.

(ii) Testing of additional fish species may be required in Tier III in order to obtain additional information on host spectrum. The type and number of species to be tested shall be determined following consultation with the Agency.

(iii) Fish species likely to prey upon or scavenge the target host organisms should be tested, when applicable.

(iv) Testing of young fish (3-6 months old) is preferable. Very young (not yet actively feeding), spawning, or recently spawned

(v) Fish should weigh between 0.5 and 5.0 grams and be from the same year class. The standard length of the longest fish should be no more than twice that of the shortest fish.

(3) Method of pesticide administration. (i) The test substance should be administered as a suspension directly into the water (i.e., aqueous exposure).

(ii) Two additional methods of pesticide administration should be considered and used in combination with the aqueous exposure in the same test, whenever possible. The two methods are:

(A) Dietary administration: food to be administered in the form of target host organisms infected with the microbial agent or feed supplemented with microbial agent; and

(B) Administration by intraperitoneal injection.

(4) Treatment concentrations. (i) If the test substance produces a toxin, then a sufficient number of treatment concentrations should be tested to determine toxicity as described in paragraphs (b)(5)(ii)(A),(B) and (b)(5)(iii) of this section.

(ii) If the test substance does not produce a toxin, or no toxin has been identified, then a single, replicated, maximum hazard exposure may be tested. Treatment concentrations or doses should be selected as follows, whenever possible:

(A) At a minimum, the concentration in the test water (for aqueous exposure) should, whenever possible, equal the maximum calculated pesticide concentration in a six-inch layer of water immediately following a direct application to a six-inch layer of water;

(B) Feed used in the dietary exposure should be supplemented with the test substance to achieve a microbial concentration greater than or equal to the host equivalent; and

(C) The injected test substance should contain a concentration of active ingredient equal to the adjusted host equivalent.

(iii) The fish infectivity test(s) conducted in Tier III may require the use of lower treatment concentrations and/or a greater number of treatment concentrations in order to determine the concentration (or dose) response relationship or the minimum effective concentration.

(5) Determination of toxicity or pathogenicity. (i) The pathogenicity of the test substance on the test organisms following a sufficiently long period of exposure and observation should be determined.

(ii) If the test substance produces a toxin, then the following shall be determined:

(A) A precise LC_{50} value with 95 percent confidence intervals; or

(B) That the LC_{50} is greater than 100 mg/l or 1,000 times the estimated environmental concentration, whichever is higher.

(iii) If data are submitted to satisfy either criterion in paragraph (b)(5)(ii)(B) of this section, the data should be derived from a study containing at least 30 organisms tested at concentrations equal to or greater than the applicable criterion (100 mg/l or 1,000x estimated environmental concentration).

(c) Reporting and evaluation of data. In addition to information meeting the general reporting requirements of § 150-3 of this subdivision, a report of the results of a fish toxicity and infectivity test would include the following:

(1) LC_{50} data (if the test substance produces a toxin).

(i) Such data should show:

(A) The 96-hour LC_{50}, the corresponding 95 percent confidence intervals, and, when possible, the LC_{50} values at 24 hour intervals for the duration of the test; or

(B) That the LC_{50} is greater than 1,000 times the expected environmental concentration or 100 mg/l.

(ii) If the data submitted in accordance with paragraph (c)(1)(i)(B) of this section indicate that the LC_{50} is greater than 1,000 times the expected environmental concentration of the pesticide, then the basis for calculating the estimated environmental concentration should be shown.

(2) Detailed description of the steps taken to determine microorganism dissemination, replication, or survival in the test animals tissues, organs, or fluids:

(3) Detailed description of dilution water, including:

(i) Source;

(ii) Chemical characteristics (e.g., dissolved oxygen content, pH, dissolved salts); and

(iii) Pretreatment (if any);

(4) Detailed description of the test, including:

(i) Design;

(ii) Containers;

(iii) Medium (e.g., depth and volume);

(iv) Treatments;

(v) Method of exposing fish to the test substance (e.g., placing microbial agent in water which already contains fish or placing fish in water which already contains the microbial agent);

(vi) Number of organisms per treatment;

(vii) Loading (weight of organisms per unit volume of medium or unit surface);

(viii) Lighting, acclimation, and test temperatures (averages and range);

(ix) Amount of test substance administered by each route of exposure; and

(x) Any unusual feature of the test method;

(5) Detailed descriptions of methods (or references to established methods) used for all chemical analyses of water for chemical content and toxicant concentrations;

(6) Detailed description of methods used for all microbial analyses of water and test organisms, and results of such analyses, including validation studies;

(7) Detailed description of the effects of exposure to the test substance including:

(i) The criteria used to determine the effects;

(ii) Percentages of organisms that died or showed effects of treatment; and

(iii) A summary of these observations; and

(8) Any additional relevant information about the test or its results that would assist in the determination of hazard potential.

(d) Tier progression. (1) If toxic or pathogenic effects are observed, then testing at Tier II [environmental expression testing (§§ 155-15 through -20)] is required as specified by 40 CFR § 158.165.

(2) Further tier testing is not necessary if results of this study do not indicate toxic or pathogenic effects.

(e) References. The following may contain useful background information for developing acceptable protocols:

(1) Anonymous. 1975. Standard Methods for Examination of Water and Wastewater. 14th Ed. American Public Health Assoc., Washington, D.C. 1193 pp.

(2) ASTM Standard E 729-80, Practice for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians. American Society for Testing and Materials, 1916 Race Street, Philadelphia, PA 19103.

(3) Committee on Methods for Toxicity Tests with Aquatic Organisms. 1975. Methods for acute toxicity tests with fish, macroinvertebrates, and amphibians. U.S. Environmental Protection Agency, Ecol. Res. Series, EPA 660/3-75-009. 61 pp.

(4) Hetrick, F.M., M.D. Knittel, and J.L. Fryer. 1979. Increased susceptibility of rainbow trout to infectious hematopoietic necrosis virus after exposure to copper. Appl. and Envir. Microb. 37(2):198-201.

(5) Huang, E., and J.S. Pagano. 1977. Nucleic acid hybridization technology and detection of proviral genomes. Chapter 13 in The Atlas of Insect and Plant Viruses, K. Maramorosch, Ed. Academic Press, New York.

(6) Ignoffo, C.M. et al. 1973. Susceptibility of aquatic vertebrates and invertebrates to the infective stage of the mosquito nematode Reesimermis nielseni. Mosquito News 33(4):599-602.

(7) Macek, K.J., S.R. Petrocelli, and B.H. Sleight III. 1979. Considerations in assessing the potential for, and significance of, biomagnification of chemical residues in aquatic food chains. ASTM STP, pp.251-268, American Society for Testing and Materials, Philadelphia.

(8) Mazzone, H.M., and G.H. Tignor. 1976. Insect viruses: serological relationships. Adv. in Virus Res. 20:237-270.

(9) Pagano, J.S., and E. Huang, 1974. The application of RNA-DNA cytohybridization to viral diagnostics. In: Viral Immunodiagnosis. E. Kurstak and R. Morisset, eds. Academic Press, Inc.

(10) Pound, J.F. 1977. Safety and potential hazards of the entomopathogen Mattesia trogodermae to nontarget species. Ph.D. Dissertation. U. Wisconsin.

(11) Reynolds, G.J. 1978. Enzyme labelled antibody in histopathology. Qualityline (Winter 1978/1979):2-10.

(12) Smith, C.E. and M.D. Summers. 1978. Analysis of baculovirus genomes with restriction endonucleases. Virology 89:517-527.

(13) Summers, M., R. Engler, L.A. Falcon, and P. Vail, eds. 1975. Baculoviruses for Insect Pest Control: Safety Considerations. Selected papers from EPA-USDA Working Symposium, American Society for Microbiology Washington, D.C.

(14) Undeen, A.H., and J.V. Maddox. 1973. The infection of nonmosquito hosts by injection with spores of the microsporidan Nosema algerae. J. Invert. Path. 22:258-265.

(15) Van Essen, F.W., and D.W. Anthony. 1976. Susceptibility of nontarget organisms to Nosema algerae (Microsporida: Nosematidae), a parasite of mosquitoes. J. Invert. Path. 28:77-85.

(16) Weber, C.E. (ed.) 1973. Biological field laboratory methods for measuring the quality of surface waters and effluents. U.S. Environmental Protection Agency, Environ. Monit. Series, EPA-670/4-73-001.

§ 154-20 Freshwater aquatic invertebrate toxicity and pathogenicity testing: Tier I.

(a) When required. Data on pathogenicity or toxicity (or both, when applicable) to an aquatic invertebrate are required by 40 CFR § 158.165 to support the registration of each end-use product intended for outdoor application and each manufacturing-use product that legally may be used to formulate such an end-use product. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Test standards. Data should be derived from tests that satisfy the general test standards in § 150-3 of this subdivision and the following test standards:

(1) Test substance. (i) Data to support the registration of end-use products and manufacturing-use products shall be derived from tests conducted with:

(A) The technical grade of each active ingredient in the product when the test substance is added directly to the test water (aqueous exposure); and

(B) The most challenging form (in terms of pathogenicity and toxicity) of each active ingredient (microorganism) in the product, when the test substance is administered in the diet or by injection.

(ii) In addition, data from testing with the end-use product are required to support the registration of any end-use product if an ingredient in the end-use product other than the active ingredient is expected to:

(A) Enhance the toxicity or pathogenicity of the active ingredient;

(B) Enhance the ability of the active ingredient (microorganism) to survive or replicate in an aquatic environment; or

(C) Independently cause toxicity to aquatic organisms.

(2) Test organisms. (i) Testing should be performed on one or two aquatic invertebrate species, depending upon the site of pesticide application as follows:

(A) Terrestrial application: test one species.

(B) Direct application into a freshwater environment: test two species.

(C) Direct application into an estuarine or marine environment: test one species.

(ii) Testing of additional aquatic invertebrate species may be required in Tier I as specified in paragraph (d)(3)(i) of this section.

(iii) Aquatic invertebrate species likely to prey upon or scavenge the diseased target host organisms should be tested, when applicable.

(iv) Immature invertebrates should be used whenever possible.

(3) Method of pesticide administration. (i) The test substance should be administered as a suspension directly into the water (i.e., aqueous exposure).

(ii) Two additional methods of pesticide administration should be considered and used in combination with the aqueous exposure in the accompanying tests, whenever possible. The two methods are:

(A) Dietary administration: feed to be administered in the form of target host organisms infected with the microbial agent or feed supplemented with microbial agent; and

(B) Administration by injection.

(4) Treatment concentrations. (i) If the test substance produces a toxin, then a sufficient number of treatment concentrations must be tested to permit a determination of toxicity as described in paragraphs (b)(5)(ii)(A), (B), and (b)(5)(iii) of this section.

(ii) If the test substance does not produce a toxin, or no toxin has been identified, then a single, replicated, maximum hazard exposure may be tested. Treatment concentrations or doses shall be selected as follows:

(A) At a minimum, the concentration in the test water (for aqueous exposure) should, whenever possible, equal the maximum calculated pesticide concentration in a six-inch layer of water, immediately following a direct application to a six-inch layer of water;

(B) Feed used in the dietary exposure should be supplemented with the test substance to achieve a microbial concentration greater than or equal to the host equivalent, whenever possible.

(C) The injected test substance should contain, whenever possible, a concentration of active ingredient equal to the adjusted host equivalent.

(5) Determination of toxicity or pathogenicity. (i) Satisfactory data should establish whether the test substance had a pathogenic or toxic effect on the test organisms during a sufficiently long period of exposure and observation.

(ii) If the test substance produces a toxin, then satisfactory data must establish either:

(A) A precise EC_{50} or LC_{50} value with 95 percent confidence intervals; or

(B) That the EC_{50} or LC_{50} is greater than 100 mg/l or 100,000 times the estimated environmental concentration, whichever is higher.

(iii) If data are submitted to satisfy either criterion in paragraph (b)(5)(ii)(B) of this section, the data should be derived from a study containing at least 30 organisms tested at concentrations equal to or greater than the applicable criterion (100 mg/l or 1,000 times the estimated environmental concentration).

(c) Reporting and evaluation of data. In addition to information general reporting requiremets of § 150-4 of this subdvision, a report of the results of an aquatic invertebrate toxicity and infectivity test would include the following:

(1) EC_{50} or LC_{50} data (if the test substance produces a toxin).

(i) Such data should show:

(A) The EC_{50} or LC_{50}, the corresponding 95 percent confidence intervals, and when possible, the EC_{50} or LC_{50} values at 24-hour intervals for the duration of the test; or

(B) That the EC_{50} or LC_{50} is greater than 1,000 times the expected environmental concentration or 100 mg/l, whichever is higher.

(ii) If the data submitted in accordance with paragraph (c)(1)(i)(B) of this section indicate that the LC_{50} or EC_{50} is greater than 1,000 times the expected environmental concentration of the pesticide, then the basis for calculating the estimated environmental concentration should be shown.

(2) Detailed description of the steps taken to determine microorganism dissemination, replication, or survival in the test animal tissues, organs, or fluids.

(3) Detailed description of dilution water, including source, chemical characteristics (e.g., dissolved oxygen content, pH, dissolved salts), and pretreatment (if any).

(4) Detailed description of the test, including:

(i) Design;

(ii) Container;

(iii) Medium (e.g., depth and volume);

(iv) Treatments;

(v) Method of exposing organisms to the test substance (e.g., placing chemical in water which contains organisms or placing organisms in water which contains chemical);

(vi) Number of organisms per treatment;

(vii) Lighting, acclimation, and test temperatures (averages and range);

(viii) Amount of test substance administered by each route of exposure; and

(ix) Any unusual feature of the test method.

(5) Detailed descriptions of methods (or references to established methods) used for chemical analyses of water for chemical content and toxicant concentrations.

(6) Detailed descriptions of methods used for all microbial analyses of water and test organisms, and the results of such analyses, including validation studies.

(7) Detailed description of the effects of exposure to the test substance, including:

(i) The criteria used to determine the effects;

(ii) Statement of percentages of organisms that died or showed effects of treatment; and

(iii) A summary of these observations.

(8) Any additional relevant information about the test or its results that would assist in the determination of hazard potential.

(d) Tier progression. (1) If toxic or pathogenic effects are observed, then testing at Tier II [environmental expression (§§ 155-15 through -20)] shall be required specified in 40 CFR § 158.165.

(2) If no toxic or pathogenic effects are observed, then no further testing at higher tiers is ordinarily necessary, except as noted in paragraph (d)(3) of this section.

(3) If host spectrum or beneficial insect tests indicate a broad host spectrum such that susceptibility of aquatic invertebrates is indicated, then either:

(i) Additional aquatic invertebrate species must be tested as described in paragraphs (a) through (c) of this section; or

(ii) Testing at Tier II, environmental expression (§§ 155-15 through -20) is required.

(4) If toxic or pathogenic effects are observed in tests conducted in accordance with paragraph (d)(3)(i) of this section, then testing at Tier II [environmental expression (§§ 155-15 through -20)] is required. If not, then no further tier testing is necessary.

(e) References. The following references may contain useful background information for developing acceptable protocols:

(1) Anonymous. 1975. Standard Methods for Examination of Water and Wastewater. 14th Ed. American Public Health Assoc., Washington, D.C. 1193 pp.

(2) ASTM Standard E 729-80, Practice for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians. American Society for Testing and Materials, 1916 Race Street, Philadelphia, PA 19103.

(3) Committee on Methods for Toxicity Tests with Aquatic Organisms. Methods for Acute Toxicity Tests with Fish, Macroinvertebrates, and Amphibians. U.S. Environmental Protection Agency, Ecol. Res. Series, EPA 660/375-009. 61 pp.

(4) Huang, E., and J.S. Pagano. 1977. Nucleic acid hybridization technology and detection of proviral genomes. Chapter 13 in The Atlas of Insect and Plant Viruses, K. Maramorosch, ed. Academic Press, N.Y.

(5) Ignoffo, C. M. _et al_. 1973. Susceptibility of aquatic vertebrates and invertebrates to the infective stage of the mosquito nematode, _Reesimermis nielseni_. _Mosquito News_ 33(4):599-602.

(6) Lightner, D.V., R.R. Proctor, A.K. Sparks, J.R. Adams, and A.M. Heimpel. 1973. Testing Penaeid shrimp for susceptibility to an insect Nuclear Polyhedrosis virus. _Environ. Entomology_ 2(4):611-613.

(7) Mazzone, H.M., and G.H. Tignor. 1976. Insect viruses: serological relationships. _Advances in Virus Research_ 20:237-270.

(8) Pagano, J.S., and E. Huang. 1974. The application of RNA-DNA cytohybridization to viral diagnostics. _In_: Viral Immunodiagnosis. E. Kurstak and R. Morisset, eds. Academic Press, Inc. New York.

(9) Pound, J.G. 1977. Safety and potential hazards of the entomopathogen _Mattesia trogodermae_ to nontarget species. Ph.D. Dissertation. U. Wisconsin.

(10) Reynolds, G.J. 1978. Enzyme labelled antibody in histopathology. _Qualityline_ (Winter 1978/1979):2-10.

(11) Smith, C.E. and M.D. Summers. 1978. Analysis of baculovirus genomes with restriction endonucleases. _Virology_ 89:517-527.

(12) Summers, M., R. Engler, L.A. Falcon, and P. Vail, eds. 1975. Baculoviruses for Insect Pest Control: Safety Considerations. Selected papers from EPA-USDA Working Symposium, American Society for Microbiology, Washington, D.C.

(13) Undeen, A.H., and J.V. Maddox, 1973. The infection of nomosquito hosts by injection with spores of the microsporidan Nosema algerae. J. Invert. Path. 22:258-265.

(14) Van Essen, F.W., and D.W. Anthony. 1976. Susceptibility of nontarget organisms to Nosema algerae (Microsporida: Nosematidae), a parasite of mosquitoes. J. Invert. Path. 28:77-85.

(15) Weber, C.E. (ed.) 1973. Biological field laboratory methods for measuring the quality of surface waters and effluents. U.S. Environmental Protection Agency, Environ. Monit. Series, EPA-670/4-73-001.

§ 154-21 Estuarine and marine animal toxicity and pathogenicity tests: Tier I.

(a) When required. Data on pathogenicity and/or toxicity to estuarine and marine animals are required by 40 CFR § 158.165 to support the registration of each end-use product intended for direct application into the estuarine or marine environment or expected to enter this environment in significant concentrations because of expected use or mobility pattern and of each manufacturing-use product that legally may be used to formulate such an end-use product. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Test standards. Data should be derived from tests that satisfy the general test standards in § 150-3 of this subdivision and the following test standards:

(1) Test substance. (i) Data to support the registration of an enduse product and a manufacturing-use product shall be derived from tests conducted with:

(A) The technical grade of each active ingredient in the product when the test substance is added directly to the test water; and

(B) The most challenging form (in terms of pathogenicity and toxicity) of each active ingredient (microorganism) in the product, when the test substance is administered in the diet or by injection.

(ii) In addition, data from testing with the end-use are required to support the registration of any end-use product if an ingredient in the end-use formulation other than the active ingredient is expected to:

(A) Enhance the toxicity or pathogenicity of the active ingredient; or

(B) Enhance the ability of the active ingredient (microorga-

(2) Test organisms. (i) Toxicity and pathogenicity should be determined for one species of shrimp and one estuarine or marine fish species.

(ii) Testing of additional estuarine or marine animal species may be required in Tier I as specified in paragraph (d)(3)(i) of this section.

(iii) Estuarine or marine animals likely to prey upon or scavenge the diseased target host organisms should be tested, when applicable.

(iv) Testing of young fish (3 to 6 months old) and immature invertebrates is preferable. Very young (not yet actively feeding), spawning, or recently spawned fish should not be tested.

(v) Fish should weigh between 0.5 and 5.0 grams and be from the same year class. The standard length of the largest fish should be no more than twice that of the shortest fish.

(3) Method of pesticide administration. (i) The test substance should be administered as a suspension directly into the water (i.e., aqueous exposure).

(ii) Two additional methods of pesticide administration should be considered and used in combination with the aqueous exposure in the same test, whenever possible. The two methods are:

(A) Dietary administration: food to be administered in the form of target host organisms infected with the microbial agent or feed supplemented with microbial agent; and

(B) Administration by injection.

(4) Treatment concentrations. (i) If the test substance produces a toxin, then a sufficient number of treatment concentrations must be tested to determine toxicity as described in paragraphs (b)(5)(ii) and (iii) of this section.

(ii) If the test substance does not produce a toxin, or if no toxin has been identified, then a single, replicated, maximum hazard exposure may be tested. Treatment concentrations or dose should be selected as follows:

(A) At a minimum, the concentration in the test water (for aqueous exposure) should, whenever possible, equal the maximum calculated pesticide concentration in a 6-inch layer of water immediately following a direct application to a 6-inch layer of water.

(B) Feed used in the dietary exposure should be supplemented with the test substance to achieve a microbial concentration greater than or equal to the host equivalent, whenever possible.

(C) The injected test substance should contain, if possible, a concentration of active ingredient equal to the adjusted host equivalent.

(iii) The estuarine or marine organism toxicity and infectivity tests conducted in Tier III may require the use of lower treatment concentrations and/or a greater number of treatment concentrations in order to determine a concentration response relationship or minimum effective concentration.

(5) Determination of toxicity, or pathogenicity and infectivity. (i) Satisfactory data must establish whether the test substance had a toxic or pathogenic effect on the test organisms during a sufficiently long period of exposure.

(ii) If the test substance produces a toxin, then satisfactory data must establish either:

(A) A precise EC50 or LC50 value with 95 percent confidence intervals; or

(B) That the EC_{50} or LC_{50} is greater than 100 mg/l or 1,000 times the estimated environmental concentration, whichever is higher. If data are submitted to satisfy either criterion in this paragraph, the data should be derived from a study containing at least 30 organisms tested at concentrations equal to or greater than the applicable criterion (100 mg/l or 1,000x the estimated environmental concentration).

(c) Reporting and evaluation of data. In addition to information meeting the general requirements of § 150-4 of this subdivision, a report of the results of estuarine or marine animal toxicity and pathogenicity tests would include the following:

(1) EC_{50} or LC_{50} data (if the test substance produces a toxin).

(i) Such data should show:

(B) That the LC_{50} or EC_{50} is greater than 1,000 times the expected environmental concentration or 100 mg/l, whichever is higher.

(ii) If the data submitted in accordance with paragraph (c)(1)(i)(B) of this section indicate that the LC_{50} or EC_{50} is greater than 1,000 times the expected environmental concentration of the pesticide, then the basis for calculating the estimated environmental concentration should be shown.

(2) A detailed description of the steps taken to determine microorganism dissemination, replication or survival in the test animal tissues, organs, or fluids;

(3) Detailed description of dilution water, including source, chemical characteristics (e.g., dissolved oxygen content, pH, dissolved salts), and pretreatment (if any);

(4) Other pertinent details, including:

(i) Design;

(ii) Containers;

(iii) Medium (e.g., depth and volume);

(iv) Treatments;

(v) Method of exposing organisms to the test substance (e.g., placing test substance in water which contains organisms or placing organisms in water which contains the test substance);

(vi) Number of organisms per treatment;

(vii) Loading (weight of organisms per unit volume of medium or unit of surface);

(viii) Lighting;

(ix) Acclimation and test temperatures (average and range);

(x) Salinities; (xi) Amount of test substance administered by each route of exposure; and

(xii) Any unusual feature of the test;

(5) Detailed description of methods (or references to established methods) used for all chemical analyses of water for chemical content and toxicant concentrations;

(6) Detailed description of methods used in all microbial analyses of water and test organisms, and the results of such analyses, including validation studies.

(7) Detailed description of the effects of exposure to the test substance, including:

(i) The criteria used to determine the effects;

(ii) A statement of the percentage of organisms that died or showed effects from the treatment; and

(iii) A summary of these observations.

(8) Any additional relevant information about the test or its results that would assist in the determination of hazard potential.

(d) Tier progression. (1) If toxic or pathogenic effects are observed, then testing at Tier II [environmental expression (§§ 155-15 through -20)] is required as specified in 40 CFR § 158.165.

(2) If no toxic or pathogenic effects are observed, then no further testing at higher tiers is ordinarily necessary, except as noted in paragraph (d)(3) of this section.

(3) If efficacy or beneficial insect tests indicate a broad host spectrum such that susceptibility of estuarine or marine invertebrates is indicated, then either:

(i) Additional estuarine or marine invertebrate species must be tested as described in paragraphs (a) through (c) of this section; or

(ii) Testing at Tier II [environmental expression (§ 155-15 through -20)] is required.

(4) If toxic or pathogenic effects are observed in tests conducted in accordance with the requirements of this section, then testing at Tier II [environmental expression (§ 155-15 through -20)] is required. Otherwise, no further tier testing is necessary.

(e) References. The following may contain useful background information for developing acceptable protocols:

(1) Anonymous. 1975. Standard Methods for Examination of Water and Wastewater. 14th Ed. American Public Health Assoc., Washington, D.C. 1193 pp.

(2) ASTM Standard E 729-80, Practice for Conducting Static Acute Toxicity Tests with Larvae of Four Species of Bivalve Molluscs.

American Society for Testing and Materials, 1916 Race Street, Philadelphia, PA 19103.

(3) Anonymous. 1978. Bioassay Procedures for the Ocean Disposal Permit Program. U.S. Environmental Protection Agency, Office of Research and Development. EPA-600/9-78-010. 121 pp.

(4) Bahner, L.H., C.D. Craft, and D.R. Nimmo. 1975. A saltwater flow-through bioassay method with controlled temperature and salinity. Prog. Fish-Cult. 37(3):126-129.

(5) Clark, J.R., and R. L. Clark, eds. 1964. Seawater systems for experimental aquariums. U.S. Dept. Int., Fish, and Wild.Serv., Bur. Sport. Fish. Wild. Res. Rep. No. 63, 192 pp.

(6) Committee on Methods for Toxicity Tests with Aquatic Organisms. 1975. Methods for acute toxicity tests with fish, macroinvertebrates, and amphibians. U.S. Environmental Protection Agency, Ecol. Res. Series, EPA 660/3-75-009. 61 pp. (Marine and estuarine species listed in this publication are acceptable.)

(7) Couch, J.A., M.D. Summers, and L. Courtney. 1975. Environmental significance of baculovirus infections in estuarine and marine shrimp. Annals N.Y. Acad. Sci. 219:528-536.

(8) DeBen, E.A. 1970. Design and construction of saltwater environment simulator. Fed. Water Qual. Admin., Pacific N.W. Water Lab., Working Paper 71:1-30.

(9) Hetrick, F.M., M.D. Knittel and J.L. Fryer. 1979. Increased susceptibility of rainbow trout to infectious hematopoetic necrosis virus after exposure to copper. Appl. Environ. Micro. 37(2):198-201.

(10) Huang, E. and J.S. Pagano. 1977. Nucleic acid hybridization technology and detection of proviral genomes. Chapter 13 in The Atlas of Insect and Plant Viruses. K. Maramorosch, ed. Academic Press, N.Y.

(11) Ignoffo, C.M., et al. 1973. Susceptibility of aquatic vertebrates and invertebrates to the infective stage of the mosquito nematode, Reesimermis nielseni. Mosquito News 33(4):599-602.

(12) Lightner, D.V., R.R. Proctor, A.K. Sparks, J.R. Adams, and A.M. Heimpel. 1973. Testing Penaeid shrimp for susceptibility to an insect Nuclear Polyhedrosis virus. Environ. Entomol. 2(4):611-613.

(13) Macek, K.J., S.R. Petrocelli, and B.H. Sleight III. 1979. Considerations in assessing the potential for, and significance of biomagnification of chemical residues in aquatic food chains.

ASTM STP, pp. 251-268, American Society for Testing and Materials, Philadelphia.

(14) Mazzone, H.M., and G.H. Tignor, 1976. Insect viruses: serological relationships. *Adv. in Virus Res.* 20:237-270.

(15) Pagano, J.S., and E. Huang, 1974. The application of RNA-DNA cytohybridization to viral diagnostics. *In*: Viral Immunodiagnosis. E. Kurstak and R. Morisset, eds., Academic Press, Inc., N.Y.

(16) Pound, J.G. 1977. Safety and potential hazards of the entomopathogen *Mattesia trogodermae* to nontarget species. Ph.D. Dissertation. U. Wisconsin.

(17) Reynolds, G.J. 1978. Enzyme labelled antibody in histopathology. *Qualityline* (Winter 1978/1979):2-10.

(18) Shelbourne, H.E. 1962. Experimental seawater systems for rearing fish larvae. Pp.81-93 *in* Seawater Systems for Experimental Aquariums. J.R. Clark and R.L. Clark, eds. U.S. Dept. Int., Fish. Wild. Serv., Bur. Sport Fish. Wild. Res. Rep. No.63. 192 pp.

(19) Smith, C.E. and M.D. Summers, 1978. Analysis of baculovirus genomes with restriction endonucleases. *Virology* 89:517-527.

(20) Strickland, J.D.H., and T.R. Parsons. 1968. A practical handbook of seawater analysis. Fish Res. Board Can. Bull. No. 167., 311 pp.

(21) Summers, M., R. Engler, L.A. Falcon, and P. Vail, eds. 1975. Baculoviruses for Insect Pest Control: Safety Considerations. Selected papers from EPA-USDA Working Symposium, American Society for Microbiology Washington, D.C.

(22) Undeen, A.H., and J.V. Maddox. 1973. The infection of nomosquito hosts by injection with spores of the microsporidan *Nosema algerae*. *J. Invert. Path.* 22:258-265.

(23) Van Essen, F.W., and D.W. Anthony. 1976. Susceptibility of nontarget organisms to *Nosema algerae* (Microsporida: Nosematidae), a parasite of Mosquitoes. *J. Invert. Path.* 28:77-85.

(24) Weber, C.E. (ed.) 1973. Biological field laboratory methods for measuring the quality of surface waters and effluents. U.S. Environmental Protection Agency, Environ. Monit. Series, EPA-670/4-73-001.

(25) White, D.B., R.R. Stickney, D. Miller, and L.H. Knight. 1973. Seawater systems for aquaculture of estuarine organisms at the Skidaway Institute of Oceanography. Ga. Mar. Sci. Center, Technical Rep. Ser. No. 73-1. 18 p.

(26) Wood, L. 1975. A controlled condition system (CCS) for continously flowing seawater. Limnol. Oceanogr. 10:475-477.

§ 154-22 Plant studies: Tier I.

(a) When required. (1) General. Data on the toxic or other adverse effects of a pesticide organism on plant growth and development are required by 40 § CFR § 158.165 to support the registration of each end-use product intended for outdoor application and each manufacturing-use product that legally may be used to formulate such an end-use product. See 40 § CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(2) Test required. The intended tests of Subdivision J should be conducted for the following use patterns:

(i) Plant-controlling pesticide organisms. The target area plant toxicity test (§ 121-1), and the growth and reproduction of vegetative vigor tests (§ 122-1), and the growth and reproduction of aquatic plants test (§ 122-2) of Subdivision J should be performed where the pesticide organism is used to control plant growth and development.

(ii) Animal-controlling pesticide organisms. The seed germination/seedling emergence and vegetative vigor test (§ 122-1) and the growth and reproduction of aquatic plants test (§ 122-2) should be performed except where:

(A) The material occurs naturally in the area of intentional usage; and

(B) The level does not exceed the naturally occurring concentration.

(b) Test standards. The plant studies as outlined in this section should meet the Subdivision J general test standards (§ 120-3) and specific test standards [§§ 121-1(b), 122-1(b), and 122-2(b)] for the appropriate tests with the following exceptions.

(1) Test substance. A typical end-use product shall be tested.

(2) Dose levels. One concentration level equal to no less than the maximum label rate shall be tested. The phrase "the maximum label rate" means the amount of active ingredient in the recommended quantity of carrier, such as water to be used per land area or applied directly to the surface of a 15-cm or 6-inch column of water.

(3) Additional plants. In addition to the plant species identified in Subdivision J (§ 122-1 and -2), five species of the same genus or, if not available, of the same family should be tested in order to evaluate the selectivity of the microbial agent. The species should be of economic importance such as horticultural or agronomic crops, or vegetation useful to domestic or wild animals.

(c) Reporting. In addition to the general information outlined in Subdivision J [§ 120-4(b)], the reporting requirements for the other tests [§§ 121-1(c), 122-1(c), and 122-2(c)] should be followed.

(d) Tier progression. (i) If an adverse effect or response on plant growth and development is 25 percent or greater for terrestrial plants and 50 percent or greater for aquatic plants with respect to the control, testing at Tier II (Environmental Expression, §§ 155-15 through 155-23) is required as specified in 40 CFR § 158.165.

(ii) If less than a 25 percent adverse effect or response for terrestrial plants or 50 per cent for aquatic plants is noted, no additional testing at higher tiers is ordinarily necessary. The Agency, after review ing the data, may recommend certain additional tests to determine a more accurate no observed effect level.

§ 154-23 Nontarget insect testing for toxicity/pathogenicity to insect predators and parasites: Tier I.

(a) When required. Data on the toxicity/pathogenicity of a microbial pest control agent are required by 40 CFR § 158.165 to support the registration of each end-use product containing a microbial pest control agent intended for outdoor application when the proposed use pattern indicates that insect predators and/or parasites may be exposed to the pesticide, and each manufacturing-use product that legally may be used to formulate such an end-use product. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Test standards. In addition to satisfying the applicable general test standards outlined in § 150-3 of this subdivision, this study should meet the following standards:

(1) Test substance. Data must be derived from testing conducted with the technical grade of each active ingredient in the product;

(2) Test species. Testing should be performed on three species of insects, representing three of the following groups:

Predaceous hemipterans
Predaceous coleopterans
Predaceous mites
Predaceous neuropterans
Parasitic hymenopterans

(3) Controls. A concurrent control group is recommended and should be treated with microbe-free (or non-viable microbe) material from the culture system used for propagation of the microbial pest control agent; and

(4) Duration of test. Control and treated insects should be observed for at least 30 days after dosing.

(c) Reporting and evaluation of data. The reporting provisions are the same as those specified in § 150-4 of this subdivision.

(d) Tier progression. (1) Non-genetically engineered microorganisms. (i) Data derived from Tier I testing will be used in conjunction with available information on use pattern, host range, and other similar factors, to assess potential for adverse effects. If data indicate potential for adverse effects, Tier II testing will be required as specified in 40 CFR § 158.165.

(ii) If toxic or pathogenic effects are not observed in this study, additional testing is ordinarily not necessary.

(2) Genetically-engineered microorganisms. Testing at the Tier II level is recommended for all genetically-engineered microorganisms, regardless of the outcome of results in Tier I.

§ 154-24 Honey bee toxicity/pathogenicity test: Tier I.

(a) When required. Data on the toxicity/pathogenicity of a microbial pest control agent are required by 40 CFR § 158.165 to support the registration of each end-use product intended for outdoor application when the proposed use pattern indicates that honey bees may be exposed to the pesticide, and for each manufacturing-use product that legally may be used to formulate such an end-use product. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Test standards. In addition to satisfying the applicable general test standards outlined in § 150-3 of this subdivision, this study shall meet the following standards:

(1) Test substance. Data must be derived from testing conducted with the technical grade of each active ingredient in the product.

(2) Test species. Testing shall be performed on the honey bee, *Apis mellifera*.

(3) Age. Test insects should be worker bees of uniform age.

(4) Controls. A concurrent control group is recommended and should be treated with microbe-free (or non-viable microbe) material from the culture system used for propagation of the microbial pest control agent.

(5) Duration of test. Control and treated bees should be observed for at least 30 days after dosing.

(c) Reporting and evaluation of data. The reporting requirements are the same as those specified in § 150-4 of this subdivision.

(d) Tier progression. (1) Non-genetically engineered microorganisms. (i) Data derived from Tier I testing will be used in conjunction with available information on use pattern, host range, and other factors, to assess potential for adverse effects. If data indicate that the potential for adverse effects exists, Tier II testing will be required as specified in 40 CFR § 158.165.

(ii) If toxic or pathogenic effects are not observed in this study, additional testing is ordinarily not necessary.

(2) Genetically-engineered microorganisms. Testing at the Tier II level is recommended for all genetically-engineered microorganisms, regardless of the outcome of Tier I testing.

(e) References. The following references are provided for use in the development of acceptable test protocols for conducting a honey bee toxicity/pathogenicity test with a microbial pest control agent:

(1) Davidson, W.W., H.L. Morton, J.O. Moffett, and S. Singer. 1977. Effect of *Bacillus sphaericus* strain SSII-1 on honey bees, *Apis mellifera*. *J. Invert. Pathol*. 29:344346.

(2) Menapace, D.M., R.R. Sackett, and W.T. Wilson. 1978. Adult honey bees are not susceptible to infection by *Nosema locustae*. *J. Econ. Entomol*. 71:304-306.

(3) Morton, H.L., J.O. Moffett, and F.D. Stewart. 1975. Effect of alfalfa looper nuclear polyhedrosis virus in honey bees. *J. Invert. Pathol*. 24:139-140.

Group B-2: Tier III Testing.

§ 154-25 Terrestrial wildlife and aquatic organism toxicity testing: Tier III.

(a) When required. The data outlined in section series 71 and 72 of Subdivision E are required by 40 CFR § 158.165 to support the registration of each end-use product and each manufacturing-use product that may be legally used to formulate such an end-use product when toxic effects on nontarget terrestrial wildlife or aquatic organisms are reported in one or more Tier I tests (§§ 154-16 through -21) and results of Tier II tests (section series 155) indicate exposure of the microbial agent to the affected nontarget terrestrial wildlife or aquatic organisms. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption who, as a general rule, is responsible for submission of the required data.

(b) Test standards. The test standards are the same as those found in §§ 71-1 through -5 and 72-1 through -6 of Subdivision E.

(c) Reporting and evaluation of data. The reporting and evaluation provisions are the same as those found in §§ 71-1 through -5 and 72-1 through -6 of Subdivision E.

(d) Tier progression. Further testing shall be required as specified in 40 CFR § 158.165 and outlined in §§ 71-1 through -5 and 72-1 through -6 of Subdivision E.

§ 154-26 Long-term avian pathogenicity and reproduction test: Tier III.

(a) When required. Data on the long-term avian pathogenicity and reproduction effects of a microbial pest control agent are required by 40 CFR § 158.165 to support the registration of each end-use product intended for outdoor application and each manufacturing-use product that legally may be used to formulate such an end-use product when:

(1) Pathogenic effects are observed in Tier I (§§ 154-16 and -17) at a level equal to the adjusted host equivalent amount; or

(2) Chronic, carcinogenic, or teratogenic effects are reported in tests outlined by §§ 152-53, -54, and -56, respectively, for evaluating hazard to humans and domestic animals; and

(3) Environmental expression testing (§§ 155-15 through -20) indicates that exposure of terrestrial animals to the microbial agent is likely.

(4) See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Test standards. Data should satisfy the general test standards in § 150-3 of this subdivision, and the following test standards:

(1) Test substance. Data shall be derived from testing conducted with the technical grade of each active ingredient in the product.

(2) Species. Testing should be performed on one avian species (preferably the bobwhite quail or mallard duck). The species selected should be the same as that selected for the avian injection pathogenicity test in § 154-17.

(3) Age. Birds approaching their first breeding season should be used.

(4) Controls. A concurrent control group is recommended and should be treated with the technical grade of the active ingredient containing inactivated microbial agent.

(5) Concentration levels. At least two treatment levels should be used. The test concentrations should include an actual or expected field residue exposure level and a multiple of that level such as 5x.

(6) Number of birds per treatment group. Each treatment group should be replicated. For bobwhite quail and mallard ducks, a minimum of 12 pen replicates should be used.

(7) Duration of exposure. Birds should be exposed to treated diets beginning not less than 10 weeks before egg laying is expected, and extending throughout the laying season.

(c) Reporting and evaluation of data. In addition to the information specified in § 150-4 of this subdivision, the test report should contain the following information:

(1) Test results. The following information should be reported for all test groups:

(i) All observed abnormal behavior;

(ii) All observed morphological and physiological responses;

(iii) Time and date of mortalities;

(iv) Results of gross necropsy tests conducted on all birds dying before termination of the test and on a representative sample of those that survived;

(v) Any evidence of multiplication of microbes (e.g., lesions) in selected body tissues that would normally be affected by an infection including the liver, kidney, lungs, spleen, cerebrospinal system and gastrointestinal tract.

(vi) Description of the method chosen to assess the cause and effect of any lesions noted;

(vii) Morbidity;

(viii) Accidental deaths or injuries;

(ix) Observable clinical signs; and

(x) Clinical tests.

(2) Test conditions. The following information should be reported for treated and untreated test groups:

(i) Species;

(ii) Strain;

(iii) Age;

(iv) Body weight;

(v) Number of birds per test (include sex ratio);

(vi) Individual identification of birds;

(vii) Diet;

(viii) Storage;

(ix) Feed consumption (grams per day);

(x) Observation on palatability or repellency;

(xi) Housing conditions of test birds:

(A) Space allocations for mating, nesting;

(B) Measurements taken to insure that the birds were protected from injuries;

(C) Lighting program, including hours per day and wattage or footcandles at bird level;

(xii) Diagram of test layout:

(xiii) Temperature;

(xiv) Water supply; and

(xv) Pretest and test history or medical and chemical administration.

(3) Egg and hatching data. The following information should be reported for each treated and untreated test group:

(i) Egg shell thickness;

(ii) Cracked eggs;

(iii) Eggs laid (number eggs per bird per day and per season);

(iv) Hatching egg storage data:

(A) Temperature;

(B) Humidity;

(C) Incubation data;

(D) Eggs set; and

(E) Egg-turning frequency;

(v) Fertility (viable embryos);

(vi) Live 3-week embryos;

(vii) Embryos that mature, embryos that pip shell, embryos that liberate themselves, and a determination of hatchability;

(viii) Dead embryos;

(ix) Fourteen-day-old survivors;

(x) Crippled survivors;

(xi) Post-hatchling mortality;

(xii) Weights of fourteen-day-old survivors; and

(xiii) Any signs of pathogenic effects in post-hatchling survivors.

(4) Pesticide test data. The levels of concentration of the microbial pest control agent in the feed and the rationale for choosing such levels should be reported.

(d) Tier progression. (1) If pathogenic effects are observed at actual or expected exposure levels:

(i) The applicant should reconsider the proposed registration of the product; and

(ii) The Agency will, at this time, review all the data and determine if a decision regarding acceptability for registration should be made. Testing at Tier IV, simulated or actual field testing (§ 154-33) may not be feasible. If adequate constraints or quarantine methods are possible, testing at Tier IV is required as specified by 40 CFR § 158.165.

(2) If no pathogenic effects are observed at actual or expected field residue exposure levels, no additional testing is ordinarily necessary.

(e) References. The following references are provided for use in the development of acceptable test protocols for conducting long-term avian pathogenicity and reproduction tests with microbial pest control agents:

(1) Heinz, G. H. 1976. Methylmercury: Second year feeding effects on mallard reproduction and duckling behavior. J. Wildl. Manag. 40(1):82-90.

(2) Heinz, G.H., and L.N. Locke. 1976. Brain lesions in mallard ducklings from parents fed methylmercury. Avian Diseases 20(1):9-17.

(3) U.S. Environmental Protection Agency, Registration of Pesticides in the United States: Proposed Guidelines, Subpart E--Hazard Evaluation: Wildlife and Aquatic Organisms. 1978 (July 10). Fed. Reg. 43(132):2972929731.

§ 154-27 Definitive aquatic animal pathogenicity tests: Tier III.

(a) When required. Data from definitive pathogenicity tests with fish and/or aquatic invertebrates are required by 40 CFR § 158.165 to support the registration of each end-use product intended for use in water or expected to be transported to water from the intended use site, and when pathogenicity or infectivity was observed in Tier I tests and to support the registration of each manufacturing-use product that legally may be used to formulate such an end-use product. See 40 CFR § 158.50 and § 158.165 to determine whether there data must be submitted; Section II-B of this sudivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Test standards. Data should be derived from tests that satisfy the general test standards in § 150-3 of this subdivision, and the test standards in Tier I (§§ 154-19 and -20), with the following exceptions:

(1) Test substance. Data should be derived from testing conducted with the most challenging form of each active ingredient (microorganism) in the product, as determined from results of Tier I testing.

(2) Test organisms. Testing should be conducted on one or more of the following types of species depending upon results of

Tier I tests, intended use sites, and estimated environmental concentrations:

(i) Freshwater fish (e.g., rainbow trout);

(ii) Freshwater aquatic invertebrate;

(iii) Estuarine or marine fish (e.g., sheepshead minnow); and/or

(iv) Estuarine or marine invertebrate (e.g., shrimp).

(3) Method of pesticide administration. The test substance should be administered either as a suspension in the test water (aqueous exposure) and/or in the diet as determined from results of Tier I tests.

(c) Reporting and evaluation of data. The provisions in Tier I, §§ 154-19 and -20, apply.

(d) Tier progression. (i) If pathogenic effects are observed, further testing at Tier IV (§ 154-29) may be specified in 40 CFR § 158.165.

(ii) If pathogenic effects are not observed, additional testing at higher tiers is ordinarily not necessary.

(e) References. Refer to paragraph (e) in §§ 154-20 and -21.

§ 154-28 Fish embryo-larvae studies and life cycle studies of fish and aquatic invertebrates: Tier III.

(a) When required. Data from fish embryo-larvae studies and/or fish life cycle studies and/or aquatic invertebrate life cycle studies are required by 40 CFR § 158.165 to support the registration of each end-use product intended for use in water or expected to be transported to water from the intended use site, and when pathogenicity or infectivity was observed in Tier I tests and to support the registration of each manufacturing-use product that may be legally used to formulate such an end-use product. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

satisfy the general test standards in § 150-3 of this subdivision, and the following test standards:

(1) Test substance. Data shall be conducted with the most challenging form of each active ingredient (microorganism) in the product, as determined from results of Tier I tests (§§ 154-19 and -20) or other Tier III tests (§§ 154-27, -29, and -30).

(2) Duration of test. (i) Fish embryo-larvae test. The embryolarvae test requires that aquatic organisms be exposed to the test substance during the embryo-larval phase (e.g., a fish "egg-fry" test), but not during all stages of life-cycle of one generation of the test species.

(ii) Aquatic invertebrate and fish life-cycle tests. The aquatic invertebrate and fish life-cycle tests require that the test animals be cultured in the presence of the test substance from egg to egg or from one stage of the life cycle to the same stage of the next generation.

(3) Test organisms and methods. The applicant should consult with the Agency regarding the appropriate species and test methods. The choice of species and test methods may have to be tailored to the microorganism's characteristics.

(c) Reporting and evaluation of data. In addition to the information specified in § 150-4 of this subdivision, the test report should contain the following information (when appropriate) on the nontarget test organism:

(1) Reproductive effects;

(2) Detailed records of spawning, egg numbers, fertility, and fecundity;

(3) Estimated no observed effect level;

(4) Mortality data;

(5) Statistical evaluation of effects;

(6) Locomotion, behavioral, physiological, and pathological effects;

(7) Definition of the criteria used to determine effects;

(8) Summary of observed signs of pathogenicity or other effects;

(9) Verification of micro-organism(s) responsible for any observed pathogenic effects; and

(10) Stage of life cycle in which organisms were tested.

(d) Tier progression. (1) If pathogenic effects are observed, further testing at Tier IV, at § 154-34, may be required as specified in 40 CFR § 158.165.

(2) If no pathogenic effects are observed, additional testing at higher tiers is ordinarily not necessary.

(e) References. The following may contain useful background information for developing acceptable protocols:

(1) Fish early-life stage:

(i) National Water Quality Laboratory Committee on Aquatic Bioassays. 1971. Recommended bioassay procedure for fathead minnow *Pimephales promelas* (Rafinesque) chronic tests. National Water Quality Laboratory. Duluth, Minn. 13 pp. (Revised January 1972.)

(ii) __________ 1971. Recommended bioassay procedure for brook trout *Salvelinus fontinalis* (Mitchell) partial chronic tests. National Water Quality Laboratory, Duluth, Minn. 11 pp. (Revised January 1972).

(iii) Hansen, D.J., P.R. Parrish, S.C. Schimmel, and L.R. Goodman. 1978. Lifecycle toxicity test using sheepshead minnows (*Cyprinodon variegatus*). Pp.109-116 *in* Bioassay Procedures for the Ocean Disposal Permit Program. U.S. Environmental Protection Agency, Office of Res. and Dev. EPA 600/9-78-010.

(2) Fish and aquatic invertebrate life-cycle tests:

(i) Biesinger, K.E. 1974(a). Procedure for *Daphnia magna* tests in standing system. U.S. Environmental Protection Agency, Environ. Res. Lab., Duluth, Minn.

(ii) Biesinger, K.E. 1974(b). Procedure for *Daphnia magna* chronic tests in flowing system. U.S. Environmental Protection Agency, Environ. Res. Lab., Duluth, Minn.

(iii) Hansen, D. J., P. R. Parrish, S. C. Schimmel, and L. R. Goodman. 1978. Life-cycle toxicity test using sheepshead minnows (*Cyprinodon variegatus*). Pp. 109-116 *in* Bioassay Procedures for the Ocean Disposal Permit Program. U.S. Environmental Protection Agency, Office of Res. and Dev. EPA-600/978-010.

(iv) Nimmo, D.E., T.L. Hamaker, and C.A. Sommers. 1978. Entire life-cycle toxicity test using mysids (*Mysidopsis bahia*) in flowing water. Pp. 64-68 *in* Bioassay Procedures for the Ocean Disposal Permit Program. U.S. Environmental Protection Agency, Office of Res. and Dev. EPA-600/9-78-010.

(v) Schimmel, S.C., and D.J. Hansen, 1974. Sheepshead minnow Cyprinodon variegatus: an estuarine fish suitable for chronic (entire life-cycle) bioassays. Proc. 28th Ann. Cong. S.E. Assoc. Game-Fish Comm. Pp. 392-398.

(vi) National Water Quality Laboratory Committee on Aquatic Bioassays. 1971. Recommended bioassay procedure for fathead minnow Pimephales promelas (Rafinesqui) chronic tests. National Water Quality Laboratory, Duluth, Minn. 13 pp. (Revised January 1972.)

(3) Additional information Additional information may be found in the following reference:

(i) Biesinger, K.E. 1974(c). Culturing methods for Daphnia and certain other cladocerans. U.S. Environmental Protection Agency, Environ. Res. Lab., Duluth, Minn.

§ 154-29 Aquatic ecosystem-pathogenicity tests: Tier III.

(a) When required. Data from aquatic ecosystem-pathogenicity tests are required by 40 CFR § 158.165 to support the registration of each end-use product intended for outdoor application and each manufacturing use product that legally may be used to formulate such an end-use product, if, after an analysis of the microbial agent's properties, the individual use patterns, and the results of previous nontarget organism and environmental expression tests, it is determined that use of the microbial agent may result in adverse effects on the nontarget organisms in aquatic environments, including those of the water column and bottom sediments. When a microbial pest control agent is used in or is expected to transport to water from the intended use site, major considerations for requiring these infectivity tests include, but are not limited to:

(1) Infectivity or pathogenicity demonstrated in previous testing; and

(2) Viability of the microorganism in natural waters as demonstrated in Tier II tests.

(3) See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Test standards. Specific standards will be established on a case-by-case basis. Data sufficient to satisfy the general test standards in § 150-3 of this subdivision, and the following test standards:

(1) Test substance. Data shall be derived from testing conducted with the most challenging form of each active ingredient (microorganism) in the product, as determined from results of Tier I tests or any other Tier III tests.

(2) Test organisms. (i) Following consultation with the Agency, the registration applicant should choose one or more of the following species to be used in aquatic ecosystem testing:

(A) A typical bottom-feeding fish (e.g., catfish or carp);

(B) A cold-water fish, a warm-water fish, or a marine fish (e.g., brook trout, rainbow trout, bass, bluegill, northern pike, walleye, or sheepshead minnow);

(C) Molluscs (e.g., oyster or freshwater clams);

(D) Crustaceans (e.g., *Daphnia* spp., shrimp, or cray fish); or

(E) Nymphs (e.g., mayfly).

(c) Reporting and evaluation of data. In addition to the information outlined in § 150-4 of this subdivision, specific data reporting and evaluation provisions will be established on a case-by-case basis following consultation with the Agency.

(d) Tier progression. (1) If pathogenic effects are observed then simulated and actual field testing may be required by 40 CFR § 158.165 and specified in § 154-33.

(2) If no pathogenic effects are observed, additional testing at higher tiers is ordinarily not necessary.

(e) References. The following may contain useful background information for developing acceptable protocols:

(1) Johnson, B.T., and R.A. Schoettger. 1975. A biological model for estimating the uptake, transfer, and degradation of xenobiotics in an aquatic food chain. *Fed. Regis.* 40(123):26906-26909. (June 25, 1975.)

(2) Macek, K.J., M.E. Barrows, R.F. Frasny, and B.H. Sleight, III. 1975. Bioconcentration of C^{14}-pesticides by bluegill sunfish during continuous exposure. Pp. 119-142 *in* Structure-activity correlations in studies of toxicity and bioconcentration with aquatic organisms. G.D. Veith and D.E. Konasewich, eds. Proceedings of

a Symposium, Burlington, Ontario, March 11-13, 1975. Sponsored by Standing Committee on Scientific Basis for Water Quality Criteria of the International Joint Commission's Research Advisory Board.

(3) Schimmel, S.C., J.M. Patrick, Jr., and A.J. Wilson. 1977. Acute toxicity to and bioconcentration of endosulfan by estuarine animals. Pp. 241-252 *in* Aquatic Toxicology and Hazard Evaluation. F.L. Mayer and J.L. Hamelink, eds. STP #634, American Society for Testing and Materials, Philadelphia, Pennsylvania.

§ 154-30 Special aquatic tests - tissue culture, microorganism/stress interaction tests. [Reserved]

§ 154-31 Plant studies: Tier III.

(a) When required. Data on the effects of a microbial pest control agent on plant growth and development are required by 40 CFR § 158.165 to support the registration of each end-use product intended for outdoor application and each manufacturing-use product that legally may be used to formulate such an end-use product where the material may transport from the site of application by air, soil, or water. The extent of movement will be determined by the environmental expression tests in Tier II (§§ 155-15 through -23). See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Test standards. The test standards are the same as those in Tiers II through III [§ 123-1 through § 124-2] of Subdivision J.

(c) Reporting. The reporting provisions are be the same as those in Tiers II through III [§ 123-1 through § 124-2] of Subdivision J.

(d) Tier progression. The tier progression criteria are the same as those in Tiers II through III [§ 123-1 through § 124-2] of Subdivision J.

§ 154-32 [Reserved]

Group B-3: Tier IV Testing.

§ 154-33 Simulated and actual field testing for mammals and birds: Tier IV.

(a) When required. (1) Data on the avian and mammalian pathogenicity of a microbial pest control agent in the field are required by 40 CFR § 158.165 to support the registration of each end-use product intended for outdoor application and each manufacturing-use product that legally may be used to formulate such an end-use product when:

(i) Pathogenic effects at actual or expected field residue exposure levels are reported in Tier III; and

(ii) The Agency determines that quarantine methods will prevent the microbial pest control agent from contaminating areas adjacent to the test area.

(2) The Agency will determine on a case-by-case basis which test (simulated small-pen field, simulated large-pen field test, or full-scale field test) shall be required.

(3) See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Test standards. Data should be derived from tests that satisfy the general test standards in § 150-3 of this subdivision and the following test standards:

(1) Test substances. Data shall be derived from testing conducted with a typical end-use product.

(c) Reporting and evaluation of data. In addition to the information specified in § 150-4 of this subdivision, the test report should contain any additional information recommended following consultation with the Agency.

(d) References. The following references are provided for use in the development of acceptable test protocols for conducting simulated and actual field tests for mammals and birds with microbial pest control agents:

(1) *Simulated large and small pen field tests*.

(i) Black, C. T., and G. L. Zorb. 1965. Effect of malathion sprays on penned pheasants. Mich. Conserv. Dept. Research and Develop. Rpt. No. 34.

(ii) Heezen, K. L. 1973. Pesticide effects on pheasants. Job Complet. Rept. Proj. No. W-118-R-6, Job No. 119.1 Mich. Dept. Conserv. 21 pp.

(iii) Kreitzer, J. F., and J. W. Spann. 1968. Mortality among bobwhites confined to a heptachlor contaminated environment. *J. Wildl. Manage.* 32(4): 874-878.

(iv) Zorb, G. L. 1968. Effects of pesticides on wildlife. Job Complet. Rpt. Proj. No. W-118-R-1, Job. No.4 Mich. Dept. Conserv. 6.

(2) *Full-scale field tests for hazard to wildlife*.

(i) Buckner, C. H., P.D. Kingsbury, B. B. McLeod, K.L. Mortenson, and D. G. H. Ray. 1974. Impact of aerial treatment on non-target organisms. Algonquin Park, Ontario and Spruce Woods, Manitoba. Inf. Rep. CC-X-59, Sect. F. Chem. Control Res. Inst. Can. For. Serv., Ottawa, Ont.

(ii) Buckner, C. H., B. B. McLeod, and P. D. Kingsbury. 1975. The effect of an experimental application of nuclear polyhedrosis virus upon selected forest fauna. Rep. CC-X-101. Chem. Control Res. Inst., Can. For. Serv., Ottawa, Ont.

(iii) Ecological Research Committee. 1970. Recommendations for an international standard for a mapping method in bird census work. Pp. 49-52 *in* Sympos. on bird Census and Environ. Monit., Bull. 9 (British Trust for Ornithology. Beech Grove; Tring; Hertfordshire, England).

(iv) Emlen, J. T. 1971. Population densities of birds derived from transect count. *The Auk* 88:343.

(v) Jolly, G. M. 1965. Explicit estimates from capture-recapture data with both death and immigration stochastic model. *Biometrika* 52:225-247.

(vi) Kingsbury P. B. McLeod, and K. Mortensen. 1978. Impact of Applications of the Nuclear Polyhedrosis Virus of the Red-headed Pine Sawfly, *Neodiprion lecontei* (Fitch), on Non-target Organisms

in 1977. Report FPM-X-11. Canadian Forestry Service, Dept. of Fisheries and the Environment.

(vii) Lautenschlager, R. A., H. Rothenbacher, and J. D. Podgwaite. 1978. Response of small mammals to aerial applications of the nucleopolyhdrosis virus of the gypsy moth, Lymantria dispar. Environ. Entomol. 7(5):676-684.

(viii) McEwen, L. C., C. E. Knittle, and M. L. Richmond. 1972. Wildlife effects from grasshopper insecticides sprayed on shortgrass range. J. Range Manage. 25(3):188-194.

(ix) Swift, D. M., and N. R. French (Coordin.) 1972. Vertebrates - small mammals. Pp. 24-28 in Basic Field Data Collection Procedures for the Grassland Biome. IBP. Nat. Res. Ecol. Lab., Ft. Collins, Colo. 86 pp. (Tech. Rpt. No. 145).

§ 154-34 Simulated or actual field testing for aquatic organisms: Tier IV.

(a) When required. (1) Data from a short-term simulated field test (where confined populations are observed), and/or an actual short-term field test (where natural populations are observed) are required by 40 CFR § 158.165 to support the registration of each product and each manufacturing-use product that legally may be used to formulate such an end-use product that is likely to cause adverse short-term or acute effects, based on consideration of available laboratory data, use patterns, and exposure rates.

(2) Data from a long-term simulated field test (e.g., where reproduction and growth of confined populations are observed) and/or an actual field test (e.g., where reproduction and growth of natural populations are observed) are required if laboratory data indicate adverse long-term, cumulative, or life-cycle effects may result from intended use.

(3) See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Test standards. Data should be derived from tests that satisfy the general test standards in § 150-3 of this subdivision, and the following test standards:

(1) Test substance. Data shall be derived from testing conducted with the end-use product.

(2) Concentration analysis. The concentration of the test substance in the water should be determined at the start of the study and from samples collected periodically.

(3) Test conditions. The test conditions for conducting field tests should resemble the conditions likely to be encountered under actual use conditions. Specifically, the pesticide should be applied at the rate, frequency, and method specified on the label.

(4) Endangered species. Studies shall not be conducted in areas containing, or suspected to contain, threatened or endangered plants or animals.

(5) Residue levels. When the test substance is applied under simulated or actual field condition testing, residues should be determined in appropriate vegetation, soil, water, sediments, and other environmental components, and in selected tissues of test organisms.

(6) Other standards. Universally acceptable standards for conducting field tests are not possible because of the many mechanisms by which a microbial agent may enter, persist, and/or reproduce in the environment, and the variety of food sources and habitats that may be affected. Therefore, the standards for conducting these tests and the information that should be reported will be established following consultation with the Agency.

(c) Reporting and evaluation of data. In addition to the information outlined in § 150-4 of this subdivision, specific data reporting and evaluation provisions will be established following consultation with the Agency.

(d) References. The following may contain useful background information for developing acceptable protocols:

(1) Anonymous. 1975. Field Testing Techniques. Fed. Regis. 40(123):26909-26912 (June 25).

(2) Buckner, C. H., P. D. Kingsbury, B. B. McLeod, K.L. Mortenson, and D. G. H. Ray. 1974. Impact of aerial treatment on non-target organisms. Algonquin Park, Ontario and Spruce Woods, Manitoba. Inf. Rep. CC-X-59, Sect. F. Chem. Control Res. Inst. Can. For. Serv., Ottawa, Ont.

(3) Buckner, C. H., B. B. McLeod, and P.D. Kingsbury. 1975. The effect of an experimental application of nuclear polyhedrosis virus upon selected forest fauna. Rep. CC-X-101. Chem. Control Res. Inst., Can. For. Serv., Ottawa, Ont.

(4) Kingsbury, P., B. McLeod, and K. Mortensen. 1978. Impact of applications of the nuclear polyhedrosis virus of the red-headed

pine saw-fly, Neodiprion lecontei (Fitch), on non-target organisms in 1977. Report FPM-X-11. Canadian Forestry Service, Dept. of Fisheries and the Environ.

(5) Mulligan, F. S., C. H. Schaefer, and T. Miura. 1978. Laboratory and field evaluation of Bacillus sphaericus as a Mosquito control agent. J. Econ. Ent. 71(5):774-777.

§ 154-35 Simulated or actual field testing for insect predators and parasites: Tier IV. [Reserved]

§ 154-36 Simulated or actual field testing for insect pollinators: Tier IV. [Reserved]

Series 155: ENVIRONMENTAL FATE AND EXPRESSION GUIDELINES FOR BIORATIONAL PESTICIDES

Subseries 155A: Tier II Environmental Guidelines for Biochemical Pest Control Agents.

§ 155-1 General information.

(a) Scope. If results of Tier I testing are positive and/or the biochemical has an aquatic use pattern, then further testing is required by 40 CFR § 158.165 to evaluate potential exposure. This section series sets forth environmental fate guidline for biochemical pesticides including guidelines pertaining to the persistence of biochemicals and to the transport of biochemicals from the site of application to another site or medium. If the data indicate that significant persistence and transport of these agents in any part of the environment occurs, such that significant exposure to nontarget organisms could be expected, additional testing in Tier III is necessary.

(b) Use of data. Environmental fate data will generally be used to determine the Estimated Environmental Concentration (EEC) by performing a simple mass-balance analysis of the pesticide, taking into consideration the pesticide application parameters (i.e., rate, frequency, and site of application) following initial tests that measure transport properties. (Volatility - § 155-4, Dispenser-water leaching § 155-5, Vapor pressure § 151-17, and Water solubility - § 151-17). Where persistence testing is required (Hydrolysis - § 1559, Aerobic soil metabolism - § 155-10, and Aerobic aquatic metabolism - § 155-11, Soil photolysis - § 155-12, Aquatic photolysis - § 155-13, Adsorption/desorption - § 155-6, and Octanol/water partition coefficient - § 155-7), each of the transformation processes should be expressed as a half-life for the particular environment or a rate constant for the environmental process depending on the test. Expected environmental concentrations can then be calculated for different times using these data and the field application rate of the pesticide. Aquatic use pattern and non-dispenser pesticides will require mass balance analysis following persistence tests.

(c) Assaying for degradation of biochemicals. Environmental fate requirements include identification of degradation products comprising more than ten percent of the initial pesticide concentration in the following studies: hydrolysis, photodegradation (soil, water, and vapor phase), and aerobic soil metabolism. When these studies are required in tests of biochemical pesticides, the very low initial pesticide concentration and the complexity of the expected products may make identification difficult.

(1) The following method is suggested for obtaining a suitable sample of the degradation products. The environmental fate studies outlined in §§ 155-9, -10, -12, or -13 should be performed at one additional high concentration. That concentration of pesticide should correspond to the highest concentration(s) required in the human health effects Tier I tests. Increasing the concentrations greatly in environmental fate studies may change the reaction mechanism in some cases (e.g., second order effects). To avoid this possibility, the rate constants (or half-lives, if the rate constants were not determined) of the reactions at high concentration should agree within expected experimental error with the rate constants/half-lives of the reactions at standard concentration. The determination should be run to approximately 90 percent reaction completion or 30 days, whichever occurs first. If there is a difference in the two rates that exceeds the error expected for the particular reaction, a different reaction resulting in different breakdown products may be occurring. This method may not be used in those situations. If the rates agree, the degradation mixture obtained may be tested in Tier I of the human health effects scheme.

(d) Monitoring for disappearance of biochemicals. The Agency will allow biomonitoring for disappearance of hormones and semiochemicals in lieu of standard instrumental analysis. Biomonitoring is useful for quantities at the detection limits of standard methods. Procedures will vary significantly with sites and pests, however, in all cases, a standard curve should be run to calibrate the method. The references listed below contain information for developing a test method.

(e) Approach. Environmental fate testing should be conducted according to the following scheme (see Figure 11):

(1) If the biochemical has an aquatic or a combined terrestrial and aquatic use pattern, the following tests should be performed. If there is significant adverse exposure, Tier III testing (§ 154-13) should be performed.

(i) Octanol/water partition coefficient (§ 155-7).

(ii) Hydrolysis (§ 155-9).

(iii) Aerobic aquatic metabolism (§ 155-11).

(iv) Aquatic photolysis (§ 155-13).

(2) If the biochemical has a terrestrial use pattern only and is not applied in a controlled release device, the provisions of paragraph (e)(2)(ii) of this section apply.

(i) If the biochemical has a terrestrial use pattern only and is applied in a controlled release device, the Volatility (§ 155-4) and Dispenser/water leaching (§ 155-5) tests should be performed. If any of the following conditions are met, then the provisions of paragraph (e)(2)(ii) of this section apply:

(A) EEC is greater than LC50 for terrestrial animals.

(B) EEC is greater than EC25 for terrestrial plants.

(C) EEC is greater than 1/5 LD50 for terrestrial animals.

(D) EEC is greater than 1/5 EC50 for insects.

(E) The biochemical leaches significantly from dispenser and Tier I tests were positive for aquatic plants and/or animals.

(ii) If Tier I test results are positive for insects only and exposure is solely through the vapor phase, the Ultraviolet-visible absorption (§ 155-8) test should be performed. If there is significant adverse exposure, Tier III (§ 154-15) testing should be performed.

(iii) If Tier I test results are not positive for insects only and exposure is not primarily through the vapor phase, then the Volatility (§ 155-4) [if not performed before], Adsorption/desorption (§ 155-6), Octanol/water partition coefficient (§ 155-7), and Hydrolysis (§ 155-9) tests should be performed.

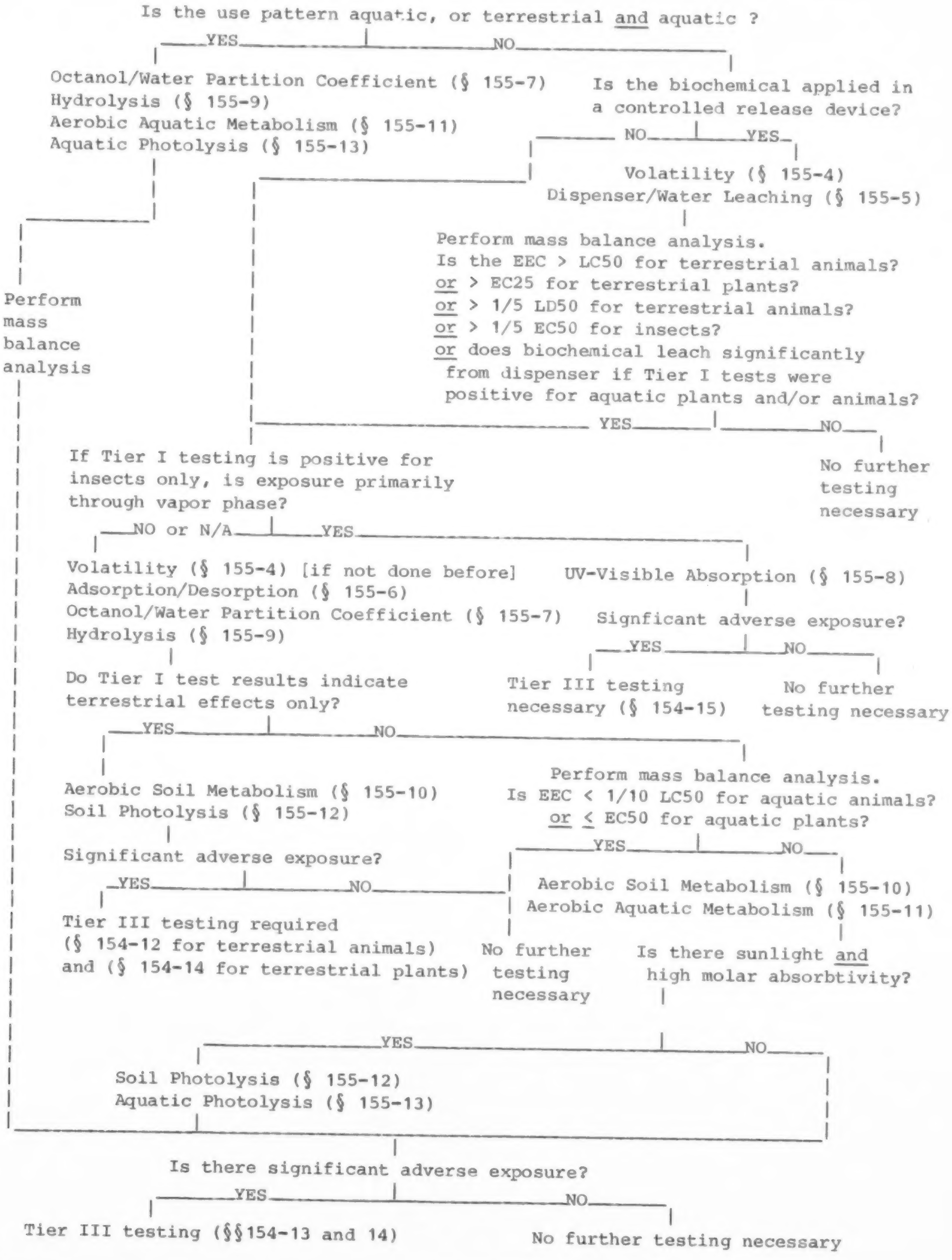
(iv) If adverse effects observed in Tier I are terrestrial effects only, the Aerobic soil metabolism (§ 155-10) and Soil photolysis (§ 155-12) tests should be performed. If there is significant adverse exposure, Tier III testing (§ 154-12 for terrestrial animals or § 15414 for terrestrial plants) should be performed. If significant adverse exposure is not found, then further testing is not necessary.

(v) If Tier I tests indicate aquatic effects and the EEC is greater than 1/10 LC50 for aquatic animals or the EEC is greater than EC50 for aquatic plants then the Aerobic soil metabolism (§ 155-10) and Aerobic aquatic metabolism (§ 155-11) tests should be performed. If not, no further testing is necessary.

(vi) If exposure to sunlight is expected and molar absorbtivity is high, then the Soil photolysis (§ 155-12) and Aquatic photolysis (§ 155-13) tests should be performed.

(vii) If there is significant adverse exposure, Tier III testing (§§ 154-13 and -14) is necessary.

Figure 11--SUMMARY OF ENVIRONMENTAL FATE TESTING SCHEME FOR BIOCHEMICALS



Is the use pattern aquatic, or terrestrial and aquatic ?

YES

Octanol/Water Partition Coefficient (§ 155-7)
Hydrolysis (§ 155-9)
Aerobic Aquatic Metabolism (§ 155-11)
Aquatic Photolysis (§ 155-13)

Perform mass balance analysis

NO

Is the biochemical applied in a controlled release device?

NO

YES

Volatility (§ 155-4)
Dispenser/Water Leaching (§ 155-5)

Perform mass balance analysis.
Is the EEC > LC50 for terrestrial animals?
or > EC25 for terrestrial plants?
or > 1/5 LD50 for terrestrial animals?
or > 1/5 EC50 for insects?
or does biochemical leach significantly from dispenser if Tier I tests were positive for aquatic plants and/or animals?

YES

NO

No further testing necessary

If Tier I testing is positive for insects only, is exposure primarily through vapor phase?

NO or N/A

Volatility (§ 155-4) [if not done before]
Adsorption/Desorption (§ 155-6)
Octanol/Water Partition Coefficient (§ 155-7)
Hydrolysis (§ 155-9)

YES

UV-Visible Absorption (§ 155-8)

Signficant adverse exposure?

YES

Tier III testing necessary (§ 154-15)

NO

No further testing necessary

Do Tier I test results indicate terrestrial effects only?

YES

Aerobic Soil Metabolism (§ 155-10)
Soil Photolysis (§ 155-12)

Significant adverse exposure?

YES

Tier III testing required
(§ 154-12 for terrestrial animals)
and (§ 154-14 for terrestrial plants)

NO

NO

Perform mass balance analysis.
Is EEC < 1/10 LC50 for aquatic animals?
or ≤ EC50 for aquatic plants?

YES

No further testing necessary

NO

Aerobic Soil Metabolism (§ 155-10)
Aerobic Aquatic Metabolism (§ 155-11)

Is there sunlight and high molar absorbtivity?

YES

Soil Photolysis (§ 155-12)
Aquatic Photolysis (§ 155-13)

NO

Is there significant adverse exposure?

YES

Tier III testing (§§154-13 and 14)

NO

No further testing necessary

(f) References.

(1) American Institute of Biological Sciences (AIBS). 1977 and 1978. Analysis of Specialized Pesticide Problems of Invertebrate Control Agents - Efficacy Test Methods. Volumes 1, 2, 3, 4, 8,and 10. EPA contracts 540/10-77-001, 540/10-77-007, 540/10-77-002, 540/10-77006, and 540/10-78-002, respectively.

(2) Arn, H., E. Stadler, and S. Raucher. 1975. The electroantennographic detector - A selective and sensitive tool in the gas chromatographic analysis of insect pheromones. Zeitschrift Naturforschung 30:772-725.

(3) Mitchell, J.W., and G. A. Livingston. 1968. Methods of Studying Plant Hormones and Growth-Regulating Substances. Agriculture Handbook No.336. Agricultural Research Service. USDA. Washington, D.C.

§§ 155-2 and -3 [Reserved]

§ 155-4 Volatility.

(a) When required. Data on the volatility of a biochemical pesticide in the environment are required by 40 CFR § 158.165 to support the registration of every end-use product intended for outdoor application and each manufacturing-use product that legally may be used to formulate such an end-use product whenever results of any one or more of the Tier I tests (§§ 154-6 through 11) indicate potential adverse effects on nontarget organisms and the biochemical agent is is to be applied on land. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who as a general rule, is responsible for submission of the required data.

(b) Test standards. In addition to the general test standards specified in § 160-4 of Subdivision N, the following specific test standards apply:

(1) Test substance. This study shall be performed using a typical end-use product.

(2) Test procedure. Samples should be tested by combined laboratory and field studies with volatility measurements taken at sufficient intervals to determine 90 percent loss of the agent from the dispenser or substrate. Field studies should be conducted under typical meteorological conditions for the environment of expected use pattern.

(c) *Reporting and evaluation of data*. In addition to the general reporting and evaluation provisions outlined in § 160-5 of Subdivision N, the following apply:

(1) Meteorologic conditions (temperature, relative humidity, wind velocity and direction, and cloud cover) during the times of exposure should be summarized. Data should be reported as micrograms product volatilized per unit time per milligram product originally applied.

(d) *Tier progression*. Further testing may be required as described in § 155-1(e) and required by 40 CFR § 158.165.

(e) *Details of method and reference*. (1) The following is an example of an acceptable protocol for conducting a volatility measurement of a semiochemical.

> Samples of the formulated product (microcapsules, tapes, etc.) should be placed outdoors in an area similar to the intended site(s) of application. Samples should be collected at five intervals beginning at the time the material is placed and continuing until 90 percent of the product is lost from the container or 90 days, whichever comes first. To determine this point, extract the product remaining in the dispenser with an appropriate organic solvent and quantitate the product in the extract in comparison with the amount of product that can be extracted from a duplicate dispenser which has not been exposed to the environment. In addition, samples of the dispenser should be laboratory tested for emission rate of the product. For example, a closed system may be arranged in a controlled temperature oven equipped with an air flow-controller allowing a flow rate of 100 ml/min, and a vapor collection device. Vapor collection time should be 2 hours. The oven temperature selected should be representative of the temperature expected at the intended site(s) of application.

(2) The following reference may provide useful background information for developing acceptable protocols:

Bierl-Leonhardt, B.A., E.D. DeVilbiss, and J.R. Plimmer. 1979. Rate of release of disparlure from laminated plastic dispensers. *J. Econ. Ent*. 72(3):319-321.

§ 155-5 *Dispenser-water leaching*.

(a) *When required*. Data on the leaching of a biochemical pesticide from a passive dispenser to the environment are required by 40 CFR § 158.165 to support the registration of every end-use product intended for outdoor application in such a dispenser and each manufacturing-use product which legally may be used to formulate such an end-use product, whenever results of any one or more of the Tier I tests (§§ 154-6 through -11) indicate potential adverse effects on nontarget organisms and the biochemical agent is to be applied on land in a passive dispenser. See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Test standards. In addition to the general test standards specified in § 160-4 of Subdivision N, the following apply:

(1) Test substance. Studies shall be performed using the end-use product when formulated for use in passive dispensers.

(2) Test procedure. This testing period should be eight hours, and the leached pesticide should be extracted from the water in which the dispenser was soaked with a suitable organic solvent. The method should be that outlined in § 163-1 of Subdivision N, except that the procedure described in § 155-1(d) may be used in place of standard instrumental analysis for monitoring disappearance of the agent.

(c) Reporting and evaluation of data. In addition to the general reporting and evaluation provisions outlined in § 160-5 of Subdivision N, the following apply:

(1) Describe any significant deviations from the protocol. Report the percentage of biochemical leached, the percentage remaining in the dispensers, and the percentage unaccounted for.

(d) Tier progression. Further tests may be required as described in § 155-1(e) and required by 40 CFR § 158.165.

(e) Details of method. The following is an example of an acceptable protocol for measuring leaching of the agent from the dispenser into water:

> Place a known amount of the formulated pesticide in unstirred water in a wide mouth container. The water volume should be large enough so that the water solubility of the active ingredient(s) will not be exceeded if all of the active ingredient(s) were to leach out of the dispensing device. Allow the pesticide/water mixture to stand for eight hours. Filter off or remove the pesticide dispenser(s) and extract the water with hexane, or some other appropriate volatile organic solvent. Dry the extract and determine the amount of semiochemical(s) either by direct measurement or after separation or concentration. At the same time, extract the dispensers that had been removed from the water, using an appropriate organic solvent. Sample this second extract

directly or after a separation or concentration step, and determine the amount of semiochemical(s) that has not leached. Calculate the percent of semiochemical(s) that leached from the dispenser.

§ 155-6 Adsorption-desorption.

(a) When required. Data on the adsorption/desorption of a biochemical pesticide in the environment are required by 40 CFR § 158.165 to support the registration of each end-use product intended for outdoor application and each manufacturing-use product which may legally be used to formulate such an end-use product, whenever results of Tier I tests indicate the need for Tier II testing as outlined in § 155-1(e). See 40 CFR § 158.50 and § 158.165 to determine whether these data must submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption who, as a general rule, is responsible for submission of the required data.

(b) Test standards. In addition to the general test standards set forth in Subdivision N in §§ 160-4 and 163-1, the following apply:

(1) Test substance. Studies shall be performed using the technical grade of the active ingredient.

(2) Test procedure. The test procedures are the same as those specified in § 163-1 of Subdivision N, except that the procedures described in § 155-1(c) may be used in place of standard instrumental analysis for monitoring disappearance of the agents when appropriate.

(c) Reporting and evaluation of data. The provisions for reporting and evaluation of data are the same as those specified in § 163-1 of Subdivision N except that a description of procedures for monitoring disappearance of the agent and corresponding test results may be patterned after the methods selected from those described in § 155-1(d).

(d) Tier progression. Further tests may be necessary as described in § 155-1(e) and required by 40 CFR § 158.165.

§ 155-7 Octanol/water partition coefficient.

(a) When required. Data on the octanol/water partition coefficient are required by 40 CFR § 158.165 to support the registration of each end-use product intended for outdoor application and each manufacturing-use product which may legally be used to formulate such an end-use product, whenever Tier I tests indicate the need for Tier II testing as outlined in § 155-1(e). See 40 CFR § 158.50 and § 158.165 to determine whether these data must submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption who, as a general rule, is responsible for submission of the required data.

(b) Test standards. The test standards are the same as those set forth in § 151-17 of this subdivision.

(c) Reporting and evaluation of data. The provisions for reporting and evaluation of data are the same as those specified in § 64-11 of Subdivision D and presented in § 151-17 of this subdivision.

(d) Tier Progression. Further tests may be necessary as described in § 155-1(e) and required by 40 CFR § 158.165.

§ 155-8 Ultraviolet-visible absorption.

(a) When required. Data on the ultraviolet-visible absorption spectra of a biochemical pesticide are required by 40 CFR § 158.165 to support the registration of each end-use product intended for outdoor application whenever results of Tier I tests (§ 154-11) indicate potential adverse effects on beneficial insects and the intended route of exposure of the pesticide is through vapor phase contact. See 40 CFR § 158.50 and § 158.165 to determine whether these data must submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption who, as a general rule, is responsible for submission of the required data.

(b) Test standards. The general test standards for environmental fate testing of biochemical pesticides are the same as those set forth in Subdivision N at § 160-4. In addition, the following specific test standards apply.

(1) Test substance. Measurements shall be performed on the analytically pure compound.

(2) Test procedure. The ultraviolet-visible absorption spectra of the active ingredients should be measured in the vapor phase at two concentrations or in the liquid phase at two concentrations. Measurements in the liquid phase should be done in non-polar, spectroscopic grade solvents such as chloroform, carbon tetrachloride, or hexane. The procedure should follow the procedures described in the 1979 Federal Register Notice (44 FR 16240), "Discussion of Premanufacture Testing Policy and Technical Issues; Request for Comment" in sections A-3.68 and A-3.69 of that document, except that the above solvent constraints apply.

(c) Reporting and evaluation of data. The reporting and calculation procedures described in 44 FR 16240, "Discussion of Premanufacture Testing Policy and Technical Issues; Request for Comment", in sections A-3.68 and A-3.69 should be followed. This calculation will provide data necessary to estimate photolytic transformation of semiochemicals in air.

(d) Tier progression. Further tests may be necessary as described in § 155-1(e) and required by 40 CFR § 158.165.

§ 155-9 Hydrolysis.

(a) When required. Data on the hydrolysis of a biochemical pesticide in the environment are required by 40 CFR § 158.165 to support the registration of every end-use product intended for outdoor application and each manufacturing-use product which may legally be used to formulate such an end-use product, whenever results of Tier I tests indicate the need for Tier II testing as described in § 155-1(e). See 40 CFR § 158.50 and § 158.165 to determine whether these data must submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption who, as a general rule, is responsible for submission of the required data.

(b) Test standards. The general test standards are the same as those set forth in Subdivision N at § 160-4. In addition, the specific test standards in § 161-1 of Subdivision N and the following standards apply:

(1) Test substance. Studies shall be performed using the technical grade of the active ingredient.

(2) Test procedure. The test procedures are the same as those specified in § 161-1 of Subdivision N, except that the procedures described in § 155-1(e) may be used in place of standard instrumental analysis for monitoring disappearance of the agent when such monitoring is necessary.

(c) Reporting and evaluation of data. The provisions for reporting and evaluation of data are the same as those specified in § 161-1 of Subdivision N, except that the procedure described in § 155-1(c) may be used to determine the toxic effects of transformation products in lieu of the identification required in this test.

(d) Tier pregression. Further tests may be necessary as described in § 155-1(e) and required by 40 CFR § 158.165.

§ 155-10 Aerobic soil metabolism.

(a) When required. Data on the aerobic soil metabolism of a biochemical pesticide in the environment are required by 40 CFR § 158.165 to support the registration of each end-use product intended for outdoor application and each manufacturing-use product which legally may be used to formulate such an end-use product, whenever results of Tier I tests indicate the need for Tier II testing as outlined in § 155-1(e). See 40 CFR § 158.50 and § 158.165 to determine whether these data must submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption who, as a general rule, is responsible for submission of the required data.

(b) Test standards. The general test standards are the same as those set forth in Subdivision N in §§ 160-4. In addition, the test standards of § 162-1 of Subdivision N and the following standards apply:

(1) Test substance. Studies shall be performed using the technical grade of the active ingredient.

(2) Test procedure. The test procedures are the same as those specified in § 162-1 of Subdivision N, except that procedures described in § 155-1(d) may be used in place of standard instrumental analysis for monitoring disappearance of the agent when necessary.

(c) Reporting and evaluation of data. The provisions for reporting and evaluation of data are the same as those specified in § 162-1 of Subdivision N, except that the approach presented in § 155-1(c) may be used in place of instrumental identification of degradable products.

(d) Tier progression. Further tests may be necessary as described in § 155-1(e) and required by 40 CFR § 158.165.

§ 155-11 Aerobic aquatic metabolism.

(a) When required. Data on the aerobic aquatic metabolism of a biochemical agent are required by 40 CFR § 158.165 to support the registration of end-use product intended for outdoor application and each manufacturing-use product that legally may be used to formulate such as end-use product whenever Tier I test results indicate the need for Tier II testing as outlined in § 155-1(e). See 40 CFR § 158.50 and § 158.165 to determine whether these data must submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption who, as a general rule, is responsible for submission of the required data.

(b) Test standards. The general test standards are the same as those set forth in Subdivision N in § 160-4. In addition, the specific test standards of § 162-4 of Subpart N and the following apply:

(1) Test substance. Studies shall be performed using the technical grade of the active ingredient.

(2) Test procedure. The procedures are the same as those specified in § 162-4 of Subdivision N except that procedures described in § 155-1(d) may be used in place of standard instrumental analysis for monitoring disappearance of the agent when necessary.

(c) Reporting and evaluation of data. The provisions for reporting and evaluation of data are the same as those specified in § 162-4 of Subdivision N, except that the approach presented in § 155-1(c) may be used in place of instrumental identification of the degradation products.

(d) Tier progression. Further tests may be necessary as described in § 155-1(e) and required by 40 CFR § 158.165.

§ 155-12 Soil photolysis.

(a) When required. Data on soil photolysis are required by 40 CFR § 158.165 to support the registraton of each end-use product intended for outdoor application and each manufacturing-use product which legally may be used to formulate such as end-use product, when Tier I test results indicate the need for Tier II testing as described in § 155-1(e). See 40 CFR § 158.50 and § 158.165 to determine whether these data must submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption who, as a general rule, is responsible for submission of the required data.

(b) Test standards. The general test standards for environmental fate testing of biochemical pesticides are the same as those set forth in Subdivision N in § 160-4. In addition, the specific test standards of § 161-3 of Subdivision N and the following apply:

(1) Test substance. Studies shall be performed using the technical grade of the active ingredient.

(2) Test procedure. The procedures are those specified in § 161-3 of Subdivision N, except that procedures described in § 155-1(d) may be used in place of standard instrumental analysis for monitoring disappearance of the agent when necessary.

(c) Reporting and evaluation of data. The provisions for reporting and evaluation of data are the same as those specified in § 161-3 of Subdivision N, except that the approach presented in § 155-1(c) may be used in place of instrumental identification of degradation products.

(d) Tier progression. Further tests may be necessary as described in § 155-1(e) and required by 40 CFR § 158.165.

§ 155-13 Aquatic photolysis.

(a) When required. Data on the aquatic photolysis of a biochemical agent are required by 40 CFR § 158.165 to support the registration of each end-use product intended for outdoor application and each manufacturing-use product which may be legally used to formulate such an end-use product whenever Tier I test results indicate the need for Tier II testing as described in § 155-1(e). See 40 CFR § 158.50 and § 158.165 to determine whether these data must submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption who, as a general rule, is responsible for submission of the required data.

(b) Test standards. The general test standards are the same as those set forth in Subdivision N in §§ 160-4. In addition, the specific test standards of § 161-2 of Subdivision N and the following specific standards apply:

(1) Test substance. Studies shall be performed using the technical grade of the active ingredient.

(2) Test procedure. The test procedure is that which is outlined in § 161-2 of Subdivision N, except that procedures described in § 155-1(d) may be used in place of standard instrumental analysis for monitoring disappearance of the agent when necessary.

(c) Reporting and evaluation of data. The provisions for reporting and evaluation of data are the same as those specified in § 161-2 of Subdivision N, except that the approach presented in § 155-1(c) may be used in place of instrumental identification of degradation products.

(d) Tier progression. Further tests may be necessary as described in § 155-1(e) and required by 40 CFR § 158.165.

§ 155-14 [Reserved]

Subseries 155B: TIER II ENVIRONMENTAL EXPRESSION DATA REQUIREMENTS FOR MICROBIAL AGENTS

§ 155-15 General information.

(a) Scope. Tier II environmental expression data are required by 40 CFR § 158.165 when toxic or pathogenic effects are observed in maximum hazard testing conducted on nontarget organisms in Tier I (§§ 154-16 through -24). The Tier II guidelines consist of tests to determine the environmental expression of a a microbial agent in a terrestrial environment (§ 155-18), in a freshwater environment (§ 155-19), and in a marine or estuarine environment (§ 155-20). These tests are intended to demonstrate whether a microbial agent is able to survive. persist or replicate in each environment, and thereby indicate which nontarget organisms will be exposed to the microbial agent, if any. A determination of the environmental expression of a microbial agent includes an evaluation of the growth of the agent when introduced into a new niche as well as an evaluation of the agents' growth pattern when its population in its normal niche is increased (as could occur immediately after application of a microbial agent). This includes normal saprophytic growth. It also includes the way a microorganism may alter its growth habits, take advantage of new environmental conditions, or take advantage of changes in the equilibrium of the microbial species which exist in a commensal association (one species benefits and the other is unaffected). Thus, the "expression" of a microorganism's presence may be through insertion into a new niche and continued propagation in the new niche.

(b) Approach. (1) Environmental expression testing consists of simulated terrestrial and aquatic applications of the microbial agent. Terrestrial applications are conducted in a greenhouse environment to assess expression in soil and vegetation. Aquatic applications are conducted in aquaria to assess expression in water and sediment.

(2) The need for terrestrial, freshwater, or marine environmental expression testing depends on:

- The Tier I test(s) in which adverse effects were observed; and
- The intended use pattern(s) for the microbial agent.

Thus, testing is only needed to assess environmental expression when susceptible nontarget species (as determined in Tier I) §§ 154-16 through -24) may be exposed.

(3) The relationship between Tier I test results, proposed use pattern, and Tier II guidelines is summarized in Table 7.

TABLE 7--SUMMARY OF ENVIRONMENTAL EXPRESSION TESTING AS DETERMINED BY TIER I TEST RESULTS AND USE PATTERNS

Tier I Test With Positive Results (Test Species)	Proposed Use Patterns for Microbial Agent		
	Terrestrial	Freshwater	Estuarine/Marine
§§ 154-16 and 17			
Avian testing - (mallard)	N/A[1]	F[3]	EM[4]
(quail)	T[2]	N/A	N/A
§ 154-18			
Mammalian testing	T	F	N/A
§§ 154-19-21			
Fish testing (freshwater sp.)	F	F	N/A
(estuarine/marine sp.)	EM	EM	EM
(estuarine/marine sp.)	EM	EM	EM
§§ 154-20,-21			
Aquatic invertebrate testing			
(freshwater ap.)	F	F	N/A
(estuarine or marine sp.)	EM	EM	EM
§ 154-22			
Terrestrial plant testing	T	N/A	N/A
§ 154-22			
Aquatic plant testing			
(freshwater sp.)	F	F	N/A
(estuaring/marine sp.)	EM	EM	EM
§§ 154-23,-24			
Terrestrial insect testing	T	N/A	N/A

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[1] N/A: Not applicable. Based on results of Tier I tests and the proposed use pattern, exposure is not expected. However, the Agency may require such tests on an individual basis.

[2] T: Tests to determine expression in a terrestrial environment are necessary.

[3] F: Tests to determine expression in a freshwater environment are necessary.

[4] EM: Tests to determine expression in a estuarine or marine environment are necessary.

(4) Genetically-engineered microbial pest control agents are subjected to a more rigorous testing scheme. Such agents should be evaluated by the applicable tests outlined in Tier II regardless of the results observed in Tier I testing. If adverse effects are observed in Tier I, testing should proceed as described in the preceding paragraph (as summarized in Table 7). However, if no adverse effects are observed, testing should be conducted in the simulated environment(s) (terrestrial, freshwater, marine or estaurine) where the exposure of nontarget organisms is expected based on the proposed use pattern.

(c) *References*. The following general references contain information useful in developing environmental expression tests for microbial agents in terrestrial, freshwater, estuarine, and marine environments as outlined in §§ 155-18 through -20:

(1) Bullock, H.R., J.P. Hollingsworth, and A.W. Hartstack. 1970. Virulence of *Heliothis* nuclear polyhedrosis virus exposed to monochromatic ultraviolet radiation. *J. Invert. Path*. 16:419-422.

(2) Burges, H.D., S. Hillyer, and D.O. Chanter. 1975. Effect of ultraviolet and gamma rays on the activity of delta-endotoxin protein crystals of *Bacillus thuringiensis*. *J. Invert. Path*. 25:5-9.

(3) Chancey, G., W.C. Yearian, and S.Y. Young. 1973. Pathogen mixtures to control insect pests. *Arkansas Farm Res*. 22:9.

(4) Forsberg, C.W., M. Henderson, E. Henry, and J.R. Roberts. 1976. *Bacillus thuringiensis*: Its Effect on Environmental Quality. Publication, National Research Council Canada. No. 15383. 135 pp.

(5) Hostetter, D.L. and C.M. Ignoffo, eds. 1977. Environmental Stability of microbial insecticides. *Misc. Publ. Entomol. Soc. Am.* 10(3): 1-117.

(6) Ignoffo, C.M., W.C. Yearian, S.Y. Young, D.L. Hostetter, and D.L.Bull. 1976. Laboratory and field persistence of new commercial formulations of the *Heliothis*-nucleopolydedrosis virus, *Baculovirus heliothis*. *J. Econ. Entomol*. 69(2):233-236.

(7) Ilnytzky, S., J.R. McPhee, and J.C. Cunningham. 1977. Comparison of field-propagated nuclear polyhedrosis virus from DouglasFir Tussock Moth with laboratory-produced virus. Pacif. For. Res. Centr. Victoria, B.C., Canada. Bi-monthly Res. Notes 33, 1. Pp. 5-6.

(8) Krieg, A. 1975. Photoprotection against inactivation of *Bacillus thuringiensis* spores by ultraviolet rays. *J. Invert. Path.* 25(2):267-268.

(9) Kalmakoff, J., and S.W. More. 1975. The ecology of nucleopolyhedrosis virus in porina (Wiseana spp.) (Lepidoptera: helialidae). New Zeal. Entomol. 6(1):73-76.

(10) Koltin, Y., and I. Chorin-Dirsch. 1971. Alteration of fungal morphology induced by a substance from Bacillus cereu. J. Gen. Microbiol. 66: 145-151.

(11) Lewis, Franklin B. 1975. Dosage effect on target insect populations (short- and long-term). Selected papers from EPA-USDA Working Symposium. M. Summers, R. Engler, L. Falcon, P. Vail, eds. American Society for Microbiology. Washington, D.C.

(12) Maddox, J.V. 1973. The persistence of Microsporida in the environment. Entomol. Soc. Amer. Misc. Publ. 9:99-104.

(13) Martignoni, M.E. and P.J. Iwai. 1977. Thermal inactivation characteristics of two strains of nucleopolyhedrosis virus (Baculovirus subgroup A) pathogenic for Orgyia pseudotsugata. J. Invert. Path. 30: 255-262.

(14) Manjunath, D. and S.B. Mathad. 1978. Temperature tolerance, thermal inactivation and ultraviolet-light resistance of nuclear polyhedrosis virus of the armyworm, Mythimna separata (Walk) (Lepidoptera; Noctuidae). (English). Zeitschrift Fur Angewandte Entomologie 87:82-90.

(15) Michael, A.H., and P.E. Nelson. 1972. Antagonistic effect of soil bacteria on Fusarium-Roseum culmorum from carnation. Phytopathology 62:1052-1056.

(16) Mitrofanov, V.B. 1976. Effect of suboptimum temperature on the activation of latent virus infection of granulosis in the codling moth. Biull. Vses Nauchno Issled Inst Zashch Rast 37:7-10. (Eng. sum.)

(17) Yendol, W.G. and R.A. Hamlen. 1973. Ecology of entomogenous viruses and fungi. Regulation of Insect Populations by Microorganisms. Ann. N.Y. Acad. Sci. 217:18-30.

(18) Young, S.Y. and W.C. Yearian. 1974. Persistance of Heliothis NPV on foliage of cotton, soybean, and tomato. Environ. Entomol. 3(2): 253-255.

§ 155-16 General test standards.

(a) Applicability. This section outlines the general test standards that apply to the studies in §§ 155-18 through -20. Applicants for registration should also comply with the specific test standards established for the particular test being conducted. In the case of conflict between general and specific test standards, the latter shall govern.

(b) Test standards. Data satisfying the provisions of § 155-17 should meet the general test standards in Subpart N (§§ 160-4 through -6), with the following exceptions:

(1) Microbial agent identification and quantification. The most specific available standard methods for the identification and quantification of the microbial pest control agent should be used. The methods used should be consistent with those in § 150-20, Product analysis.

§ 155-17 Reporting and evaluation of data.

(a) Results. (1) Data should be collected to determine whether the microbial agent is able to survive, persist or replicate in the environment under test. This data should be expressed in the form of a population growth or decline curve for the microbial agent. Any other applicable method of expressing the expression of the microbial agent population may also be used.

(2) Test reports should also contain the information designated in following list, modified as necessary to be applicable to the microbial pest control agent being tested. This information should be given in sufficient detail to adequately define growth characteristics of the test organism.

(i) pH and temperature for optimal growth, and the ranges of pH and temperature within which the microorganism can survive and grow.

(ii) Essential nutrients for growth:

(A) Carbon source (CO_2, carbonate, other);

(B) Minerals;

(C) Organic compounds; and

(D) Cofactors.

(iii) Potential for autotrophic growth (i.e., growth expected in the absence of a particular facultative growth requirement).

(iv) Growth response to variations in salinity.

(v) Response to known antagonists:

(A) Chemical;

(B) Biological; and

(C) Others.

(vi) Serological comparison with known pathogens to provide identification and/or environmental marker(s).

(vii) Biochemical characteristics that are unique to the organism.

(viii) Morphological variation in response to adverse conditions:

(A) Humidity;

(B) Inorganic or organic content of water;

(C) Salinity; and microorganism.

§ 155-18 Tests to determine expression in a terrestrial environment.

(a) When required. Data on the expression of a microbial pest control agent in a terrestrial environment are required by 40 CFR § 158.165 to support the registration of each end-use product intended for outdoor application on land and each manufacturing-use product that legally may be used to formulate such an end-use product when toxic or pathogenic effects are observed in any of the following Tier I tests:

(1) Avian single dose oral toxicity and pathogenicity test (§ 15416);

(2) Avian injection pathogenicity test (§ 154-17);

(3) Wild mammal toxicity and pathogenicity test (§ 154-18);

(4) Plant studies - terrestrial (§ 154-22);

(5) Testing for toxicity/pathogenicity to insect predators and parasites (§ 154-23); or

(6) Honey bee toxicity/pathogenicity test (§ 154-24).

(7) See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains

an additional discussion of the formulators' exemption and who, as a general rule, is responsible for submission of the required data.

(b) Test standards. (1) Method. (i) Tests shall be conducted in a greenhouse environment to determine whether the microbial agent is able to survive, persist and replicate in a terrestrial environment consisting of soil and vegetation representative of the proposed use site. The following parameters should be varied to determine their effect on the survival and growth of the microbial agent population:

(A) Temperature;

(B) Humidity;

(C) Precipitation (amount, frequency, pH);

(D) Sunlight;

(E) pH (soil and foliar surfaces);

(F) Nutrients (soil, vegetation).

(ii) The values selected for each parameter listed in paragraph (b)(1)(A) through (F) of this section should be selected to approximate the conditions expected at the intended use site.

(iii) Laboratory studies designed to determine the microbial agent's growth requirements (e.g., temperature, humidity, pH, sunlight, and nutrients) may supplement the greenhouse study described in paragraph (b)(1)(i). Laboratory studies may demonstrate that the microbial agent will be unable to sur will consider studies on a case-by-case basis to meet the intent of testing in § 155-18 in lieu of the greenhouse study.

(2) Test substance. A typical end-use product or the technical grade of the active ingredient shall be tested.

(3) Test duration. Data to establish a population decline curve shall be collected at intervals until two half-life determinations have been made or until data establish that the microbial agent population is able to maintain itself in the terrestrial environment at or above the level present immediately after test initiation.

(c) Reporting and evaluation of data. The reporting and evaluation provisions are the same as those set forth in § 155-17.

(d) Tier progression. If results of this study indicate that the microbial agent is able to persist in the terrestrial environment such that the susceptible non-target organism(s) tested in Tier I are likely to be exposed, then the appropriate testing in Tier III (§§ 154-25, -26, or -31) is required as specified in 40 CFR § 158.165.

(e) *References*. The following references contain information for developing acceptable protocols.

(1) Anthony, D.W., K.E. Savage, E.I. Hazard, S.W. Avery, M.D. Boston, and S.W. Oldacre. 1978. Field test with *Nosema algerae* Vavra and Undeen (Microsporida, Nosematidae) against *Anopheles albimanus* Wledemann in Panama. *Miscel. Publ. Entomol. Soc. Amer*. 11:17-28.

(2) Cunningham, J.C. 1970. Persistence of the nuclear polydrosis virus of the eastern hemlock looper on balsam foliage. Insect Pathology Res.Institute. Sault Ste. Marie, Ontario, Canada. *Bi-monthly Res. Notes* 26:24-25.

(3) Elgee, E. 1975. Persistence of a virus of the white-marked tussock moth on balsam fir foilage. Maritimes Forest Res. Centre. Fredericton, New Brunswick, Canada. *Bi-monthly Res. Notes* 31:33-34.

(4) Grison, P., D. Martouret, B. Servais, and M. Devriendt. 1976. Microbial pesticides and environment. *Ann. Zool. Ecol. Anim*. 8(2):133-160.

(5) Harcourt, D.J. 1968. Persistence of a granulosis virus of *Pieris rapae* in soil. *J. Invert. Path*. 11:142-143.

(6) Hukuhara, T., and H. Namura. 1972. Distribution of a nuclearpolyhedrosis virus of the fall webworm, *Hyphantria cunea*, in soil. *J. Invert. Path*. 19:308-316.

(7) Ignoffo, C.M., C. Garcia, D.L. Hostetter, and R.E. Pinell. 1978. Stability of conidia of an entomopathogenic fungus, *Nomuraea rileyi*, in and on soil. *Environ. Entomol*. 7(5):724-727.

(8) Ignoffo, C.M., G. Garcia, D.L. Hostetter, and R.E. Pinnell. 1977. Vertical movement of conidia of *Nomuraea rileyi* through sand and loam soils. *Environ. Entomol*. 7(2):270-272.

(9) Jaques, R.P. 1967b. The persistence of a nuclear-polyhedrosis virus in the habitat of the host insect, *Trichoplusia ni*. 11. Polyhedra in soil. *Can. Entomol*. 99:820-829.

(10) Jaques, R.P. 1969. Leaching of the nuclearpolyhedrosis virus of *Trichoplusia ni* from soil. *J. Invert. Path*. 13:256-263.

(11) Jaques, R.P. 1974a. Occurrence and accumulation of viruses of *Trichoplusia ni* in treated field plots. *J. Invert. Path*. 23:140-152.

(12) Jaques, R.P. 1974b. Occurrence and accumulation of the granulosis virus of Pieris rapae in treated field plots. J. Invert. Path. 23:351-359.

(13) Kerr, A. 1974. Soil microbiological studies on Agrobacterium radiobacter and biological control of crown gall. Soil Sci. 118(3):168-172.

(14) Ladd, T.L., Jr., and P.J. McCabe. 1967. Persistence of spores of Bacillus popilliae, the causal organism of Type A milky disease of Japanese beetle larvae in New Jersey soils. J. Econ. Entomol.60(2):493-495.

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§ 155-19 Tests to determine expression in a freshwater environment.

(a) When required. (1) Data on the expression of a microbial pest control agent in a freshwater environment are required by 40 CFR § 158.165 to support the registration of each end-use product intended for outdoor application on land and each manufacturing-use product that legally may be used to formulate such an end-use product when toxic or pathogenic effects are observed in any of the following Tier I tests:

(i) Freshwater fish toxicity and pathogenicity testing (§ 154-19);

(ii) Freshwater aquatic invertebrate toxicity and pathogenicity test (§ 154-20); or

(iii) Plant studies - aquatic (§ 154-22).

(2) Data on the expression of a microbial pest control agent in a freshwater environment are required by 40 CFR § 158.165 to support the registration of each end-use product intended for outdoor application on fresh water and each manufacturing-use product that legally may be used to formulate such an end-use product when toxic or pathogenic effects are observed in any of the following Tier I tests:

(i) Avian single dose oral toxicity and pathogenicity test (§ 154-16);

(ii) Avian injection pathogenicity test (§ 154-17);

(iii) Wild mammal toxicity and pathogenicity testing (§ 154-18);

(iv) Freshwater fish toxicity and pathogenicity testing (§ 154-19);

(v) Freshwater aquatic invertebrate toxicity and pathogenicity test (§ 154-20); or

(vi) Plant studies - aquatic (§ 154-22).

(3) See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision

contains an additional discussion of the formulators' exemption and who as a general rule, is responsible for submission of the required data.

(b) Test standards. (1) Method. (i) Tests shall be conducted in a simulated aquatic environment (e.g., aquarium with bottom sediment) to determine whether the microbial agent is able to survive, persist, and replicate in a freshwater environment consisting of fresh water and bottom sediment representative of the proposed use site. The following parameters should be varied to determine their effect on the survival and growth of the microbial agent population:

(A) Temperature;

(B) pH;

(C) Nutrients;

(D) Sunlight;

(E) Oxygen content;

(F) Hardness; and

(G) Turbulence.

(ii) The values selected for each parameter listed in paragraph (b)(1)(A) through (G) of this section should be selected to approximate the conditions expected at the intended use site.

(iii) Specialized laboratory studies designed to determine the microbial agent's growth requirements (e.g., temperature, pH, sunlight, oxygen) may supplement the study described in paragraph (b)(1)(i) of this section. Specialized lab studies may demonstrate that the microbial agent will be unable to survive and persist in a freshwater environment. In such instances, the Agency will consider studies on an individual basis to meet the intent of testing in § 155-19 in lieu of the study described in paragraph (b)(1)(i) of this section.

(2) Test substance. A typical end-use product or the technical grade of the active ingredient shall be tested.

(3) Test duration. Data to establish a population decline curve should be collected at intervals until two half-life determinations have been made or until data establish that the microbial agent population is able to maintain itself in a freshwater environment at or above the level present immediately after test initiation.

(c) Reporting and evaluation of data. The reporting and evaluation provisions are the same as those set forth in § 155-17.

(d) Tier progression. If results of this study and use patterns information indicate that the microbial agent is likely to enter and is able to persist in a freshwater environment such that the susceptible nontarget organism(s) tested in Tier I are likely to be exposed, then the appropriate testing in Tier III (§§ 154-25 through -31) is required as specified in 40 CFR § 158.165.

(e) References. The following references contain useful information for developing acceptable protocols.

(1) Anonymous. 1975. Impact of the use of microorganisms on the aquatic environment. EPA publication 660-3-75-001. Technical Publications Office, Environmental Protection Agency, National Environmental Res. Center, Corvallis, Oregon. 97330.

(2) Anthony, D.W., K.E. Savage, E.I. Hazard, S.W. Avery, M.D. Boston, and S.W. Oldacre. 1978. Field tests with *Nosema algerae* Vavra and Undeen (Microsporida, Nosematidae) against *Anopheles albinamus* Wiedemann in Panama. *Misc. Publ. Entomol. Soc. Amer*. 11:17-28.

(3) Brand, R.J., D.E. Pinnock, K.L. Jackson, and J.E. Milstead. 1975. Methods for assessing field persistence of *Bacillus thuringiensis* spores. *J. Invert. Path*. 25:199-208.

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§ 155-20 Tests to determine expression in a marine or estuarine environment.

(a) When required. (1) Data on the expression of a microbial pest control agent in a marine or estuarine environment are required by 40 CFR § 158.165 to support the registration of each end-use product intended for outdoor application on land or in fresh water and each manufacturing-use product that legally may be used to formulate such an end-use product when toxic or pathogenic effects are observed in any of the following Tier I tests:

(i) Estuarine and marine animal toxicity and pathogenicity test (§ 154-21); or

(ii) Plant studies - estuarine or marine (§ 154-22).

(2) Data on the expression of a microbial pest control agent in a marine or estuarine environment are required by 40 CFR 158.165 to support the registration of each end-use product intended for outdoor application in marine or estuarine environments and each manufacturing-use product that legally may be used to formulate such an end-use product when toxic or pathogenic effects are observed in any of the following Tier I tests:

(i) Avian single dose oral toxicity and pathogenicity test (§ 154-16);

(ii) Avian injection pathogenicity test (§ 159-17);

(iii) Estuarine and marine animal toxicity and pathogenicity test (§ 154-21); or

(iv) Plant studies - estuarine or marine (§ 154-22).

(3) See 40 CFR § 158.50 and § 158.165 to determine whether these data must be submitted; Section II-B of this subdivision contains an additional discussion of the formulators' exemption and who as a general rule, is responsible for submission of the required data.

(b) Test standards. (1) Method. (i) Tests shall be conducted in a simulated marine or estuarine environment (e.g., aquarium with bottom sediment) to determine whether the microbial agent is able to survive, persist, and/or replicate in a marine or estuarine environment consisting of seawater or brackish water and bottom sediment representative of the proposed use site. The following parameters should be varied to determine their effect on the survival and growth of the microbial agent population:

(A) Temperature;

(B) pH;

(C) Nutrients;

(D) Salinity;

(E) Sunlight;

(F) Oxygen content; and

(G) Turbulence.

(ii) The values selected for each parameter listed in paragraph (b)(1)(A) through (G) of this section should be selected to approximate the conditions expected at the intended use site.

(iii) Specialized laboratory studies designed to determine the microbial agent's growth requirements (e.g., temperature, pH, sunlight, oxygen) may supplement the study described in paragraph (b)(1)(i) of this section. A specialized lab study(ies) may demonstrate that the microbial agent will be unable to survive and persist in a marine or estuarine environment. In such instances, the Agency will consider this study(ies) on an individual basis to fulfill the intent of the testing in § 155-20 in lieu of the study described in paragraph (b)(1)(i) of this section.

(2) Test substance. A typical end-use product or the technical grade of the active ingredient shall be tested.

(3) Test duration. Data to establish a population decline curve should be collected at intervals until two half-life determinations have been made or until data establish that the microbial agent population is able to maintain itself in a marine or estuarine environment at or above the level present immediately after test initiation.

(c) Reporting and evaluation of data. The reporting and evaluation provisions are the same as those set forth in § 155-17. In addition, the following information should be reported:

(1) Any changes in morphology of the microorganism in response to changes in salinity.

(d) Tier progression. If results of this study and use pattern information indicate that the microbial agent is likely to enter and is able to persist in a marine or estuarine environment such that the susceptible nontarget organism(s) tested in Tier I are likely to be exposed, then the appropriate testing in Tier III (§§ 154-25 through -31) is required as specified by 40 CFR § 158.165.

(e) References. Refer to § 154-19(e).

§§ 155-21, -22, and -23 [Reserved]

Series 156: PRODUCT PERFORMANCE GUIDELINES FOR BIORATIONAL PESTICIDES

§ 156-1 General provisions.

(a) Waiver of data requirements: background and policy. The recent amendment to sec. 3(c)(5) of FIFRA provides that the Administrator may waive data requirements pertaining to efficacy. This amendment states:

> In considering an application for the registration of a pesticide, the Administrator may waive data requirements pertaining to efficacy, in which event the Administrator may register the pesticide without determining that the pesticide composition is such as to warrant proposed claims of efficacy.

The Agency, in testimony before Congress, stated that it is most concerned about ensuring a product's effectiveness when a lack of efficacy could result in adverse human health effects. In keeping with this concern, the Administrator has deemed that all applications for products not having a direct impact on public health may have their efficacy requirements waived. The Agency is limiting its direct concern to, and requiring efficacy data for, products having health related use patterns and products proposing new and added uses of chemicals which have been identified as posing a risk of unreasonable adverse effects.

(1) Efficacy data will generally only be required by 40 CFR § 158.165 for products of the following types:

(i) Uses of agents intended to control microorganisms infectious to man in any area (inanimate surface) where these microorganisms may present a health hazard; and

(ii) Uses of agents intended for control of fungal organisms that produce aflatoxins.

(2) Data on phytotoxicity to the target site, i.e., crops or other desirable plants, are considered part of an efficacy evaluation and are thus waived. [On the other hand, data on phytotoxicity to crops or other plants that are non-target sites are considered to be data for hazard evaluation and must be submitted on a case-by-case basis as prescribed in §§ 154-10 and -14. Data on the effects of microbial pest control agents on nontarget plants must be submitted for all such products as described in §§ 154-22 and -31 with cross reference to Subdivision J.]

§ 156-2 Specific provisions.

(a) The following provisions apply to all biorational pesticides regardless of whether product performance data are or are not waived in accordance with § 156-1(a):

(1) The available information on host spectrum shall be reported;

(2) The time required to achieve the desired level of pest control or other product performance standard shall be reported; and

(3) The minimum effective dosage (MED) necessary to achieve the desired level of pest control or other product performance standard shall be reported. The registrant is referred to Subdivision G, Product Performance, for specific guidance and information on data and reporting requirements.

Series 157: EXPERIMENTAL USE PERMIT GUIDELINES FOR BIORATIONAL PESTICIDES

§ 157-1 Scope and intent.

This section series deals with the data necessary to support the application for an experimental use permit for a biorational pesticide. These guidelines are based on FIFRA secs. 5 and 40 CFR Part 172, and they closely match the guidelines in Subdivision I in many respects. For further information on scope and intent, refer to § 110-1 of Subdivision I.

§ 157-2 [Reserved]

§ 157-3 General Provisions.

In developing plans and information for an experimental use permit application, the applicant should carefully review section series 110 and 111 of Subdivision I. With the exception of several cross references to specific data in other subdivisions of the guidelines, the provisions of those sections of Subdivision I apply to biorational pesticides as well as to conventional pesticides.

§ 157-4 Specific data requirements.

(a) General. (1) The following types of data are required by 40 CFR § 158.165 to support an application for an experimental use permit for a biorational pesticide:

(i) Product analysis; refer to paragraph (b) of this section;

(ii) Residues; refer to paragraph (c) of this section;

(iii) Toxicology; refer to paragraph (d) of this section;

(iv) Nontarget organisms; refer to paragraph (e) of this section;

(v) Environmental fate; refer to paragraph (f) of this section; and

(vi) Product performance; refer to paragraph (g) of this section.

(2) General policies related to data necessary to support an experimental use permit are delineated in Subdivision I, § 112-1.

(b) Product analysis data. To support an application for an experimental use permit, the data outlined in §§ 151-10 through -18 apply to biochemical pest control agents and §§ 151-20 through -26 apply to microbial pest control agents.

(c) Residue data. For biochemical pest control agents, residue data are required by 40 CFR § 158.165 to support an application for an experimental use permit when the product will be used on food or feed crops or when its use is expected to result in residues in or on food or feed and for either of the following situations:

(1) The rate of biochemical pest control agent application exceeds 50 grams active ingredient per acre per application; or

(2) Tier I toxicology studies conducted under paragraph (d) of this section or under section series 153 of this subdivision indicates a potential for human hazard. Residue data requirements will be determined on an individual basis for biochemicals applied directly to food or feed, and for biochemicals whose application rate can not be expressed in ounces per acre per application. In these situations, the data necessary to obtain a temporary tolerance (see Subdivision O, Chemistry Requirements: Residue Chemistry) are required. For microbial pest control agents used on food or feed crop or whose use is expected to result in residues in or on food or feed, no data are required unless Tier I toxicology studies conducted under section series 153 of this subdivision indicates a potential for human hazard. Residue data developed in accordance with Subdivision O would then be required to obtain a temporary tolerance.

(d) Toxicology data. The following data are required by 40 CFR § 158.165 to support an application for an experimental use permit:

(1) Biochemical pest control agents not used on food crops:

(i) Acute oral toxicity (§ 152-10);

(ii) Acute dermal toxicity (§ 152-11);

(iii) Primary eye irritation (§ 152-13);

(iv) Primary dermal irritation (§ 152-14); and

(v) Studies to detect gene mutation (§ 152-17).

(2) Biochemical pest control agents used on food crops:

(i) All studies listed in paragraph (d)(1) of this section; and

(ii) Cellular immune response studies (§ 152-18).

(3) Microbial pest control agents not used on food crops:

(i) Acute dermal infectivity (§ 152-31);

(iii) Intravenous, intracerebral, intraperitoneal infectivity (§ 152-33);

(iv) Primary dermal irritation (§ 152-34); and

(v) Primary eye irritation (§ 152-35).

(4) Microbial pest control agents used on food crops:

(i) All studies listed in paragraph (d)(3) of this section;

(ii) Cellular immune response (§ 152-37); and

(iii) Tissue culture with viral agents (§ 152-39).

(e) Nontarget organism data. To support an application for an experimental use permit, nontarget organism data developed in Tier I studies of biochemical and microbial pest control agents as described in section series 154 are required as specified in 40 CFR § 158.165.

(f) Environmental fate and expression data. To support an application for an experimental use permit, data from environmental fate and expression studies according to section series 155 are required by 40 CFR § 158.165 for those biochemical and microbial pest control agents whose Tier I nontarget organism test results (from section series 154) indicate that Tier II studies for environmental fate and expression should be conducted. For those pest control agents whose Tier I nontarget organism test results indicate no Tier II studies are necessary, no enviromental fate and expression data are required for the application of a permit. In those instances where field data from Tier II studies are required in section series 155 for a permit, any comparable or limited field data would suffice in lieu of extensive field data; this policy is needed to preclude development of extensive field data without a permit in order to obtain information necessary to get a permit.

(g) Product performance data.

(1) General. In general, efficacy data will not be required by 40 CFR § 158.165 to support the issuance of an experimental use permit.

(2) Exceptions. (i) Initial permits. Efficacy data may be required, on a case-by-case, for the following use categories:

(A) Public health uses dealing with microscopic pest organisms; and

(B) Use of cancelled or suspended pesticides.

(ii) Extensions, renewals, and amendments. Summaries of product performance data collected under an experimental use permit may be requested on a case-by-case basis by the Agency for purposes of making:

(A) Determinations as to the need for additional quantities of product requested by the applicant;

(B) Evaluations of requests for permit extensions; and

(C) Assessments of requests for permit renewals.

Series 158: LABEL DEVELOPMENT

§ 158-1 Product label requirements.

Biorational pesticides are generally subject to all applicable labeling provisions described in Subdivision H - Labeling Requirements for Pesticides and Devices. Biochemical agents are viewed essentially the same as conventional chemical pesticides with respect to label requirements, but labeling for microbial agents differ principally with respect to the ingredient statement. Some instruction regarding ingredient statements for microbial agents can be derived from §§ 151-20 through -25 (Product Analysis) of this subdivision. Also, see § 156-1(b)(6) regarding label claims, directions, precautions, and restrictions in relation to use pattern information for biorational pesticides.